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NAD^+/NADH and skeletal muscle mitochondrial adaptations to exercise

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Running title: NAD^+-mediated regulation of mitochondrial biogenesis

Abstract

The pyridine nucleotides, NAD^+ and NADH, are coenzymes that provide oxidoreductive power for the generation of ATP by mitochondria. In skeletal muscle, exercise perturbs the levels of NAD^+, NADH and consequently, the NAD^+/NADH ratio, and initial research in this area focused on the contribution of redox control to ATP production. More recently, numerous signaling pathways that are sensitive to perturbations in NAD^+(H) have come to the fore, as has an appreciation for the potential importance of compartmentation of NAD^+(H) metabolism and its subsequent affects on various signaling pathways. These pathways, which include the sirtuin (SIRT) proteins, SIRT1 and SIRT3, the poly(ADP-ribose) polymerase (PARP) proteins, PARP1 and PARP2, and C-terminal binding protein (CtBP), are of particular interest because they potentially link changes in cellular redox state to both immediate, metabolic-related changes and transcriptional adaptations to exercise. In this review we discuss what is known, and not known, about the contribution of NAD^+(H) metabolism and these aforementioned proteins to mitochondrial adaptions to acute and chronic endurance exercise.

Introduction

Nicotinamide (NAM) adenine dinucleotide (NAD^+; initially known as diphosphopyradine nucleotide [DPN^+]), is a ubiquitous cellular coenzyme that was first discovered by Arthur Harden and William Young, when they identified a heat-labile fraction of cell-free glucose fermentation containing ATP, Mg^{2+} and NAD^+, which they coined, "cozymase" (78). Our understanding of the role of NAD^+ and its reduced form, NADH, in cellular function and metabolism was subsequently expanded by a "who's who" of biochemistry, with researchers such as Hans von Euler-Chelpin, Otto Warburg, Conrad Elvehjem, Arthur Kornberg, Albert Lehninger and Britton Chance, all making substantial contributions. Four of the aforementioned researchers were awarded the Nobel Prize, with Harden and von Euler-Chelpin sharing the Nobel Prize in 1929 for their work on the fermentation of sugar and fermentative enzymes, which included the identification of the "nucleotide sugar phosphate", NAD^+. Subsequently, Warbug demonstrated that NAD^+ acted as a carrier of hydrogen and transferred it from one molecule to another, which was key to understanding the metabolic function of NAD^+ (128). Ultimately, it was work by Freidkin and Lehninger (55) that showed that NADH was an integral component of ATP production via oxidative phosphorylation. Thus, for many years the primary cellular function of NAD^+ was considered to be its ability to harness energy from glucose, fatty acids, and amino acids in pathways such as glycolysis, β-oxidation, and the citric acid cycle.

In recent years, however, the importance of NAD^+ as a central signaling molecule and substrate that can impact numerous fundamental biological processes has come to the fore. Indeed, a remarkable number of regulatory pathways that utilize NAD^+ in signaling reactions have been identified, and these cover broad aspects of cellular homeostasis including functions in energy metabolism, lifespan regulation, DNA repair, apoptosis and telomere maintenance (11, 12, 84, 97, 190). Thus, while the tissue NAD^+/NADH ratio was once thought to be 'simply' a balance of the redox state, the complexity of NAD^+ metabolism has evolved considerably with the discovery of highly integrated networks of NAD^+ consuming pathways and NAD^+ biosynthetic and salvage pathways (11, 12, 84, 97, 128, 144, 190). Part

of the reason for the renaissance of NAD^+ has been the discovery of NAD^+-consuming enzymes, particularly, sirtuins (SIRT). SIRT1 is the most well-described of the seven mammalian sirtuins, and based on its dependence for NAD^+ as a substrate (and therefore its sensitivity to perturbations in NAD^+), SIRT1 has been put forth as a key regulator of acute and chronic exercise-mediated mitochondrial adaptations in skeletal muscle (40, 70, 72, 76, 174, 185, 193). In addition, SIRT3 and poly-ADP-ribose (PAR) polymerases (PARPs), which also use NAD^+ as a substrate, have been proposed as important regulators of mitochondrial function and/or biogenesis (40, 76, 125, 174, 185, 193). In this review our aim is to provide an overview of NAD^+ metabolism in skeletal muscle and the changes that occur in NAD^+, NADH, and the NAD^+/NADH ratio in response to acute and chronic endurance exercise. Our intention is not to discuss the impact of the redox state and NAD^+/NADH ratio on cellular bioenergetics and substrate utilization, which is covered in highly informative reviews by others (9, 26, 106, 109, 110). Rather, our goal is to discuss the changes in pyridine nucleotide redox state that occur with exercise in the context of what we know and do not know about the effects of SIRT1, SIRT3, the PARPs and carboxyl-terminal binding protein (CtBP), on mitochondrial adaptations to exercise in skeletal muscle. It is of course difficult to extrapolate the findings from one cell line or tissue type to another, and we acknowledge that we do not discuss many important studies that have contributed to our understanding of NAD^+ metabolism and SIRT1, SIRT3 and PARP biology in cell lines and tissue types other than skeletal muscle and muscle cell lines. For a more general and encompassing discussion on NAD^+ metabolism and its potential clinical implications, readers are encouraged to read some excellent and comprehensive reviews (see, (11, 12, 84, 97, 128, 144, 190)).

Where in the cell is NAD^+?

It is broadly accepted that NAD^+ is primarily found in three distinct cellular pools, 1) the cytosolic, 2) the mitochondrial, and 3) the nuclear pools. A general overview of the compartmentation of NAD^+ and NADH is provided in Figure 1, and provides a point of reference for the ensuing discussion on

NAD^+(H) compartmentation and their movement into the mitochondria and nucleus. Initial studies used differential centrifugation methods, cell disruption methods, and compounds, to modulate mitochondrial NAD^+(H) metabolism in order to determine NAD^+(H) location. More recently, the 'compartmentation' of NAD^+, which was originally suggested by Ragland and Hackett (146), has been extrapolated from the localization of enzymes in the NAD^+ consuming, biosynthetic, and salvage pathways, and the use of innovative molecular biology techniques (11, 12, 84, 97, 144, 190). Thus, Dölle et al. (43) used a novel PAR Assisted Protein Localization AssaY (PARAPLAY) in HeLa S3 cells, in which they targeted the catalytic domain of PARP1 (which consumes NAD^+) to various cellular compartments. The idea behind this method is that if NAD^+ is present in the compartment to which PARP1 is targeted, then PAR will accumulate and can be detected by immunocytochemistry (43). Using PARAPLAY, NAD^+ was found in the mitochondria (specifically the matrix but not intermembrane space) and peroxisomes, and surprisingly to the endoplasmic reticulum (ER) and Gogli complex (43, 112). Cytosolic NAD^+ was not detected in this study, most likely due to the fact that PAR glycohydrolase (PARG), which consumes PAR, is most abundant in the cytosol. Little is known about the role of NAD^+ and NADH in regulating Golgi complex and ER function, and certainly its function in skeletal muscle is unknown. Furthermore, surprisingly very little is known about nuclear NAD^+ levels in general, and to our knowledge nuclear NAD^+(H) levels have not been measured in skeletal muscle. Overall, the free cytosolic and nuclear NAD^+(H) compartments are traditionally thought to be in equilibrium, with NAD^+(H) being able to freely pass through pore complexes in the nuclear membrane (46, 98-103, 187, 190). In Cos7 cells the free nuclear NAD^+ concentration is estimated to be ~10-100 μM (53, 188), which is comparable to the estimations for the cytosol (~150 μM) of muscle (42, 119). Thus, in response to exercise, it would be expected that the pyridine redox state in the nucleus reflect changes that occur in the cytosol. The relevance of nuclear NAD^+(H) to adaptations to exercise will be covered when discussing SIRT1, PARPs, and C-terminal binding protein (CtBP).

NAD^+ and NADH concentrations in skeletal muscle at rest.

While PARAPLAY provides qualitative insight into the location of NAD^+, determining the precise concentration of NAD^+ in various compartments remains challenging. Typically, absolute concentrations of NAD^+ and NADH have been calculated using biochemical and extraction methods, whilst the metabolite indicator method (MIM) has be used to extrapolate the 'free' cytoplasmic and mitochondrial NAD^+/NADH ratio by measuring the concentrations of specific cytoplasmic and mitochondrial redox couples. The MIM carries a number of assumptions, such as the selected dehydrogenase reaction being a near-equilibrium reaction and that the reaction occurs in one cellular compartment, at pH 7.0 (63, 107, 179). In skeletal muscle, the most common application of the MIM is calculation of the cytosolic free NAD^+/NADH ratio, via measurement of lactate and pyruvate levels, based on the lactate dehydrogenase (LDH) reaction (107, 179). The mitochondrial free NAD^+/NADH ratio, can be determined by measuring the concentrations of glutamate, α-ketoglutarate ,and NH_3, and is based on the glutamate dehydrogenase (GDH) reaction (107, 179), although GDH activity is low in skeletal muscle (10, 179).

In resting human muscle, total NAD^+ and NADH concentrations are estimated to be ~1.5-1.9 and ~0.08-0.20 mmol/kg dry weight (dw) muscle, respectively (62, 80, 93, 154, 155, 159, 160). Based on the approximate volumes of distributions of mitochondria, the extra-mitochondrial space (i.e., cytosol) and their mass fractions (i.e., % of cell volume: cytosol = 90% and mitochondria = 10% (50)), Cabrera and colleagues (42, 119) estimate the total, mitochondrial, and cytosolic compartment concentrations in skeletal muscle for NAD^+ and NADH, respectively, to be approximately- Total: 0.45 and 0.05 mmol/kg cell wet weight [ww]; Cytosol: 0.15 and 0.00028 mmol/kg cytosolic ww; Mitochondria: 3.15 and 0.5 mmol/kg mitochondrial ww (Note: to convert to dw muscle, multiply by ~4.2 (145)). Thus, the NAD^+/NADH ratio in resting skeletal muscle is estimated to be much higher in the cytosol (~540) as compared to mitochondria (~6.3), and overall, greater than ~95% of cellular NADH is estimated to be in the mitochondrial compartment. The nucleus comprises ~1% of muscle cell volume (50), and considering

that the nuclear-to-cytosolic NAD^+(H) levels are considered to be in equilibrium, the nuclear NAD^+ and NADH concentrations would be estimated to be comparable to the aforementioned values for the cytosol. Although higher than estimates in other cells (NAD^+: ~10-100 μM; NADH: ~130 nM (53, 188), considering the high density of mitochondria and metabolic turnover of skeletal muscle, these approximations seem reasonable.

Relevant to the redox state and covalent activation of NAD^+- or NADH-dependent signaling proteins is the fact that most cellular NAD^+ and NADH is bound to proteins (13, 54, 171, 176, 179, 180). This makes it quantitatively difficult to determine the free NAD^+ and NADH levels (and the free NAD^+/NADH ratio), which ultimately represent the metabolically active forms of these coenzymes. Measurement of free NAD^+(H) levels is further complicated by the fact that NADH binds proteins more firmly than NAD^+ (54, 171, 180). It should be noted, however, that studies in rat hippocampus using time-resolved fluorescence and anisotropy decay suggest the ratio of free-to-bound NADH to be ~0.78 (175). Whether this is the case in skeletal muscle is unknown. Based on the MIM for LDH, in resting skeletal muscle the free cytosolic NADH level is estimated to be ~0.5-1.5% of total cytosolic NADH (158).

In skeletal muscle, NAD^+ levels are highest in the mitochondria (42, 119), thus by extension one might infer that oxidative skeletal muscle (with a greater abundance of mitochondria) would have overall higher NAD^+ levels compared to glycolytic muscle. Supporting this notion, in human resting muscle, NAD^+ concentration is positively correlated with the % of slow twitch fibers (62). However, in rat soleus and extensor digitorum longus (EDL) muscles, no differences in NAD^+ levels were noted, although differences in the degree of reduction of the NAD^+ couple were found (i.e., higher NAD^+ levels in soleus vs. EDL mitochondria), which may be indicative of the differing metabolic characteristics of these muscles (158).

Changes in NAD^+ and NADH concentrations and the NAD^+/NADH ratio in muscle during exercise.

Animal studies. Early studies by Britton Chance and colleagues (27, 28, 33) and others (61, 87,

88), typically in amphibian muscle, used fluorescence-based methods (128, 129) to demonstrate that NADH levels decrease (and thus NAD^+ levels increase) during muscle contraction. With respect to mammalian muscle, Jobsis and Stainsby (89) used the same technique to study NADH oxidation in the gastrocnemius-plantaris and gracilis muscle groups in dogs, and found that low-intensity (5 Hz) and tetanic contractions increased NAD^+ levels. By manipulating the ability of mitochondria to oxidize NADH, they concluded that the increase in tissue NAD^+ primarily occurs inside mitochondria (89). In contrast to studies that show that NAD^+ increase with contraction, Duboc et al. reported an increase in NADH during tetanic contractions in soleus and EDL muscles of the rat (44). A limitation of the fluorometric technique used in these studies is that it does not provide quantitative assessment of NAD^+, NADH, and the NAD^+/NADH ratio. Addressing this limitation, Edington and colleagues (48) measured NAD^+ biochemically, and estimated the NAD^+/NADH ratio using the MIM method (using the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios). Thus, in untrained and trained rats, cytosolic and mitochondrial NAD^+ concentrations, as well as the NAD^+/NADH ratio, were increased by low-intensity muscle contraction of the gastrocnemius-plantaris muscles. As one would expect, the increase in the mitochondrial NAD^+/NADH ratio during the same absolute exercise was lower in trained rats (47, 48). In the soleus and EDL muscles of the rat, twitch or tetanic contractions increased NAD^+ levels (as measured by decreased NADH fluorescence) during contraction (178). Supporting this notion, studies in insect and canine muscle using the MIM method (based on the glutamate dehydrogenase [GDH] reaction) found that the mitochondrial NAD^+/NADH ratio is increased during exercise at a variety of exercise intensities (34, 135, 152, 153, 181). Chronic low-frequency (10 Hz) stimulation of the rat tibialis anterior muscle also increased NAD^+ levels after 15 min of contraction, and the NAD^+/NADH ratio was significantly increased for up to 24 h of stimulation (65). In mice, swimming exercise increased muscle NAD^+ levels (23), and in rats endurance exercise training resulted in a sustained (as samples were measured 2 days after the last exercise bout) increase in NAD^+ levels in gastrocnemius muscle of young and old rats (104). However, an increase in NAD^+ and the NAD^+/NADH ratio during exercise is not a

182 universal finding. In one study NADH increased and the NAD^+/NADH ratio decreased during flight in
183 insect muscle (77), whilst in mouse muscle no change in NAD^+ levels at the end of running exercise was
184 found, though an increase 3 h after exercise was noted (22). In addition, in electrically-stimulated canine
185 muscle (gastrocnemius-plantaris muscles), cytoplasmic NAD^+ levels were reduced during exercise (64),
186 whilst in electrically-stimulated (5 Hz) soleus muscle, no change in NAD^+ levels was found (167).
187 *Human studies.* In human muscle, the effects of exercise on NAD^+ levels and the NAD/NADH ratio are
188 largely the opposite of those found in animal studies. Muscle NAD^+ levels were decreased when
189 exercising at 65% and 100% of maximal oxygen uptake (VO_2max), and while increased muscle water
190 accounted for ~73% of this decrease, NAD^+ levels were still reduced when assessed on a dry weight basis
191 (62). The first studies to quantitatively measure both NAD^+ and NADH levels in human muscle at rest
192 and during exercise were conducted by Dr. Kent Sahlin and colleagues (80, 93, 154, 155, 160). During
193 maximal exercise and submaximal isometric contractions NADH increased ~140% above resting levels,
194 whereas there was no significant change in NAD^+ levels (80, 155). In contrast, no change in total muscle
195 NADH concentration was noted throughout exercise at 75% VO_2max (157), whilst NADH and the
196 cytosolic NAD^+/NADH ratio were decreased during exercise at 50% VO_2max (93). Similar to this, a
197 number of studies found that the cytosolic NAD^+/NADH ratio is reduced during exercise (66, 141),
198 although the magnitude of reduction is lower after exercise training (141). Exercise intensity appears to be
199 an important contributor to the differences in measured NAD^+(H) and NAD^+/NADH ratio during exercise
200 in animal vs. human studies. For example, NADH decreased (and the cytosolic NAD^+/NADH ratio was
201 unchanged) from resting values during exercise at 40% VO_2max, but both NAD^+ and the cytosolic
202 NAD^+/NADH ratio were increased above resting values at 75% and 100% VO_2max (160). Moreover, a
203 series of *in silico* studies (that distill the NAD^+ and NADH information from some of the aforementioned
204 papers) predict that whole tissue, cytosolic, and mitochondrial NAD^+/NADH ratios are reduced during
205 exercise at 60% VO_2max (119), but are increased during exercise at a lower intensity (65 watts) (21, 41).
206 Interestingly, estimation of the mitochondrial redox state during exercise in human muscle using the MIM

207 method, estimated that the free NAD^+/NADH ratio is significantly increased at 75% and 100% VO_2max
208 (63).

209 *Summary*. There are conflicting results in both animal and human studies as to whether or not
210 exercise increases or decreases NAD^+, NADH and the NAD^+/NADH ratio. There are many reasons that
211 may underlie these differences including training state, intensity of contraction, duration of exercise, time
212 point of measurement during exercise, the analytical technique used to measure NAD^+(H) and the
213 NAD^+/NADH ratio (e.g., fluorometric, biochemical, MIM method), and the compartment that was
214 measured (whole tissue, mitochondrial or cytosolic). From a more 'big picture' perspective, because the
215 majority of change in muscle NADH levels with exercise is presumed to occur within the mitochondrial
216 compartment, a large increase in NADH during exercise would correspond to a decreased redox potential,
217 which could be inhibitory on mitochondrial oxidative enzymes and limit TCA cycle flux (63). The
218 simplest explanation for this would be a 'backing up' of the electron transport chain (ETC) due to
219 limitations in the capacity to oxidize NADH. This is supported by the findings that elevated total muscle
220 NADH concentrations decrease to resting levels during recovery from high intensity exercise (80, 155).
221 Alternatively, an increase in the mitochondrial redox potential would be expected to facilitate generation
222 of NADH by increasing the availability of NAD^+ for pyruvate dehydrogenase and the various
223 dehydrogenase reactions of the TCA cycle and β-oxidation (63). In muscle, measurement and
224 extrapolation of NAD^+(H) metabolism during exercise is further complicated by the fact that muscle
225 comprises subsarcolemmal and intermyofibrillar mitochondria, which are known to have different
226 capacities for substrate oxidation (32, 108, 184). Whether NAD^+(H) kinetics during exercise is different
227 within these mitochondrial populations is unknown, and it is likely that fluorometric studies of NAD^+(H)
228 metabolism with contraction reflect changes in the subsarcolemmal compartment and not the 'whole'
229 muscle. Considering these results and unresolved questions as a whole, it is clear that a major gap in our
230 understanding of NAD^+(H) metabolism during exercise is that no study has directly measured the free
231 NAD^+ and NADH levels or the subcellular localization and compartmentation of NAD^+(H) metabolism.

232 Such analysis is clearly very technically challenging and will likely require the use of advanced
233 techniques such as HPLC and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry in
234 combination with tissue fractionation methods or two-photon excitation microscopy (139, 162, 182, 188).
235 Ultimately, measuring the free $NAD^+(H)$ levels is what is most important when it comes to regulation of
236 proteins and pathways responsive to perturbations in $NAD^+(H)$, such as SIRT1, SIRT3, and PARPs, and
237 subsequent effects on cellular function and metabolism.
238
239 **Shuttling of NADH into the mitochondria.**
240 The inner mitochondrial membrane is impermeable to NAD^+ and NADH (115, 143), and shuttles are
241 required to transport NADH from the cytosol to the mitochondria (138). This is accomplished via the
242 exchange of metabolites that are reduced in the cytosol and oxidized in the mitochondria (138). In skeletal
243 muscle these are the glycerol-3-phosphate (G3P; or α-glycerophosphate) shuttle and the malate-aspartate
244 (M-A) shuttle (83, 138, 163-165). Considering that exercise training enhances the capacity of muscle to
245 oxidize NADH, the activities of enzymes of the M-A shuttle are higher in trained vs. untrained muscle
246 (29, 83, 163, 165), as well as in oxidative vs. glycolytic muscle (29, 163). Moreover, muscle MDH
247 activity decreases with detraining (29). In contrast, the activity of G3P dehydrogenase, a key enzyme in
248 the G3P shuttle, is not increased by exercise training (163, 165), but is higher in glycolytic vs. oxidative
249 muscle (83, 163). Reducing equivalents may also be transferred to the mitochondria via the lactate shuttle,
250 which is explained in detail elsewhere (18, 60). Briefly, the lactate shuttle hypothesis posits that cytosolic
251 pyruvate is primarily converted to lactate, which is then transported via facilitated diffusion into the
252 mitochondria, where it is converted back to pyruvate by intramitochondrial LDH (18, 19, 60). Therefore,
253 the lactate shuttle, via the LDH reaction, would allow for transfer of NADH from the cytosol to
254 mitochondria in a manner similar to the G3P and M-A shuttles. It should be noted that as debated by
255 others, there is significant controversy over the presence of LDH within pure mitochondria and the
256 existence of a lactate shuttle in skeletal muscle mitochondria (16, 20, 59, 147, 156, 184). In recent years

the NADH/cytochrome c (cyto c) electron transport shuttle has also been described, in which the direct transfer of electrons from cytosolic NADH to molecular oxygen inside the mitochondrial matrix is achieved at respiratory contact sites (i.e., where both mitochondrial membranes are in contact) (1, 123). The transfer capacity of the NADH/cyto c is reported to be equivalent to the malate-aspartate shuttle (1, 123). However, whether this system is active in skeletal muscle mitochondria, or is regulated by exercise training, is unknown.

Mitochondrial adaptations to endurance exercise: Role of SIRT1 and SIRT3

Sirtuins are a family of class III deacetylases that possess NAD^+-dependent deacetylase and mono-ADP-ribosyltransferase activities (40, 76, 125, 174, 185, 193). Over the past decade there has been an explosion of research on the therapeutic potential of treating various diseases via activation of sirtuins, especially SIRT1, and more recently, SIRT3 (40, 76, 125, 174, 185, 193). In fact, a search on PubMed reveals that in just the past 12 years some 300 reviews have been published on sirtuins alone, with the majority of these focusing on SIRT1. The requirement of NAD^+ for the deacetylase function of SIRT1 and SIRT3 provides a fundamental link between the activity of these proteins and perturbations in NAD^+(H) status during exercise. Accordingly, our focus here is to discuss the role of SIRT1 and SIRT3 in regulating the effects of acute and chronic exercise on mitochondrial function and biogenesis. A more general overview of sirtuin biology and function can be found elsewhere (40, 76, 125, 174, 185, 193).

***SIRT1*.** SIRT1 is the most studied of the mammalian sirtuins and is mainly found in the nucleus, although it also has cytosolic targets (40, 76, 174, 185, 193). Of particular importance to the focus of this review was the discovery that SIRT1 deacetylates and positively regulates the activity of PGC1α, a master regulator of mitochondrial biogenesis (5, 57, 132, 150). Thus, SIRT1 has also been put forth as a principal regulator of mitochondrial biogenesis via its ability to regulate PGC1α function. Following this, a number of studies have noted that SIRT1 gene (31, 45, 127) or protein (68, 117, 118, 121, 122, 173) levels increase in skeletal muscle in response to acute or chronic exercise, in parallel with upregulation of

mitochondrial content. However, other studies have found either no effect (25, 75) or a decrease (73-75, 104) in SIRT1 protein in skeletal muscle with chronic muscle contraction (via electrical stimulation) or endurance exercise. Complimenting these latter studies, skeletal muscle SIRT1 protein content does not scale with muscle oxidative capacity or PGC1α abundance (73-75). Moreover, when SIRT1 was overexpressed in skeletal muscle, mitochondrial function and abundance (as measured by electron transport chain [ETC] and mitochondrial transcription factor A [mtTFA] protein abundance, citrate synthase activity), gene expression of mitochondrial proteins, and PGC1α gene and/or protein expression was not changed (56, 140) or even decreased (74). In C2C12 myotubes, overexpression of SIRT1 increased PGC1α gene expression and PGC1α promoter activity (5), although effects on mitochondrial biogenesis and function were not assessed. When SIRT1 protein (15, 22, 56, 57) or deacetylase activity (142) is knocked out in skeletal muscle of mice or C2C12 myotubes there is no reduction in mitochondrial function (e.g., O_2 consumption, proton conductance, activity of electron transport chain [ETC] enzymes or citrate synthase), number (as measured by mtDNA:nDNA ratio, ETC protein abundance), PGC1α gene and/or protein expression, or the gene expression of mitochondrial proteins. In contrast, PGC1α gene expression is lower in the TA, gastrocnemius, and soleus of SIRT1-null mice, although whether this reduction impacts PGC1α protein expression, mitochondrial biogenesis, or mitochondrial function was not assessed (5). Moreover, in studies in C2C12 and mouse primary myotubes, SIRT1 knockdown downregulates mitochondrial and fatty acid oxidation gene expression, fatty acid oxidation, and citrate synthase (CS) activity (22, 57), whilst SIRT1 overexpression increases PGC1α expression, transcriptional activity, and mitochondrial genes (5, 57). Despite reductions in PGC1α gene expression, SIRT1 knockdown in C2C12 myotubes does not reduce PGC1α protein expression (56, 57).

Possible reasons for discrepancies between these different studies have recently been reviewed (70, 72). An obvious reason for many of these differences relates to differences between studying SIRT1 biology *in vitro* using muscle cells (particularly C2C12 muscle myotubes), versus *in vivo* using rodent

models and adenovirus techniques. Also, the precise definition of mitochondrial biogenesis and function is different across these studies, with measurement of the gene expression of PGC1α and mitochondrial genes being a common outcome measure. While measurement of gene expression provides important information, if positive or negative effects on mitochondrial biogenesis/function are to be concluded, it will be helpful in future studies to provide a more complete assessment of mitochondrial biogenesis/function, which may include measurement of mitochondrial protein synthesis and turnover, submaximal and maximal O_2 consumption, ETC enzyme activity and protein abundance, the mtDNA:nDNA ratio, or mitochondrial morphology by electron microscopy.

To resolve the incongruent findings regarding SIRT1 protein levels and mitochondrial adaptations to exercise, it has been proposed that SIRT1 activity might be the underlying mediator of these changes. Nuclear SIRT1 activity is positively correlated with oxidative capacity (i.e., CS activity, complex IV abundance) across different muscle types and is also associated with the onset of mitochondrial adaptations to acute exercise, as well as chronic changes in oxidative capacity that occur with exercise training (75). Other studies have also reported an increase in SIRT1 activity (as measured by the SIRT1 activity assay or deacetylation of PGC1α) with acute and chronic muscle contraction (22, 23, 25, 73, 75, 104, 117, 118), although no increase was found with voluntary wheel running (despite increased mitochondrial biogenesis) (25). Notably, the SIRT1 activity assay uses a peptide substrate that contains Fluor de Lys, a non-physiological fluorescent moiety, and studies using this assay (25, 73-75, 104), may be complicated by the fact that measured SIRT1 activity is potentially an artifact of the fluorophore itself (17, 90). This assay also measures SIRT1 activity in the presence of maximal NAD^+, which does not reflect the NAD^+ levels in the muscle. With this in mind, measurement of the acetylation status of proposed SIRT1 targets (e.g., p53, FOXO, or PGC1α), SIRT1 binding to the promoters of known gene targets, or measurement of the gene expression of SIRT1 target genes would compliment measures of SIRT1 activity, and provide a more physiological readout of SIRT1 function.

It is important to note that SIRT1 activity can be regulated via phosphorylation (56, 69, 91, 161). Recently, Gerhart-Hines (56) demonstrated that SIRT1 was phosphorylated in its catalytic domain by protein kinase A (PKA), which is also activated by endurance exercise. In addition, activation of PKA (via forskolin) increased SIRT1 phosphorylation and activity, including induction of PGC1α expression in skeletal muscle (56). This occurred despite no increase in NAD^+ (56), perhaps suggesting that SIRT1 activity (and function) could be regulated independently of NAD^+ in skeletal muscle. However, the effects of exercise on SIRT1 phosphorylation in skeletal muscle are unknown.

A limitation of the aforementioned studies that investigate SIRT1 and exercise-induced mitochondrial biogenesis is that they are correlative, and do not address whether SIRT1 is required for exercise-induced mitochondrial biogenesis in skeletal muscle. To address this limitation, Philp et al. (142) studied the effects of acute and chronic exercise training on muscle function, PGC1α acetylation and mitochondrial biogenesis in mice with muscle-specific knockout of SIRT1 deacetylase activity (mKO^{SIRT1}). In muscle from mKO^{SIRT1} mice there was no compensatory upregulation in the gene expression of SIRT2-7 or the protein abundance of SIRT3 and SIRT6 (unpublished observations; S. Schenk, A.T. White and A. Philp). Similar to previous studies in mice (14), no impairment in mitochondrial function or number (e.g., abundance and/or activity of complexes I-IV of the ETC, CS activity, mtDNA:nDNA ratio) in muscle from mKO^{SIRT1} vs. control mice was found, nor was muscle endurance capacity impaired (142). Interestingly, mKO^{SIRT1} and control mice also had comparable reductions in PGC1α acetylation and induction of exercise-response genes (e.g., mitofusin 2, PDH kinase 4, cytochrome c) after acute exercise, and normal mitochondrial adaptations (e.g., abundance and/or activity of complexes I-IV of the ETC, CS activity, mtDNA:nDNA ratio) to wheel running training (142). Thus, studies in mKO^{SIRT1} mice reveal that SIRT1 deacetylase activity is not required for normal function of mitochondria in skeletal muscle, nor is it required for exercise-induced adaptations. Regarding PGC1α acetylation, the authors found that the acetyltransferase that regulates PGC1α transcriptional activity, general control of amino acid synthesis 5 (GCN5) (57, 116, 132), is modulated by exercise, such that

355 nuclear localization of GCN5 was reduced and less GCN5 co-immunoprecipitated with PGC1α after
356 exercise (142). Similarly, whole-body deletion of SRC-3, an upstream activator of GCN5, results in
357 decreased PGC-1α acetylation and increased mitochondrial biogenesis (36), whilst overexpression of
358 GCN5 reduces mitochondrial gene expression and fatty acid oxidation (57). This study suggests,
359 therefore, that the reduced acetylation of PGC1α with exercise is not due to increased deacetylation by
360 SIRT1, but rather is a result of reduced acetylation by GCN5 (142). This is an interesting finding, and
361 demonstrates that PGC1α acetylation is a balance of the activities of the proteins that acetylate and
362 deacetylate it. Currently, the mechanisms by which exercise regulates GCN5 activity, GCN5 translocation
363 from the nucleus, and the GCN5-PGC1α interaction, are unknown.

364 How SIRT1 gene expression is regulated in response to exercise is also unknown. In liver cells,
365 SIRT1 gene expression is regulated via opposing effects of cyclic AMP response-element-binding protein
366 (CREB) and carbohydrate response-element-binding protein (ChREBP)(134), such that increased CREB
367 binding to the SIRT1 promoter increases SIRT1 transcription, whereas ChREBP binding impairs it.
368 CREB has also been shown to regulate PGC1α transcription (3, 4). Given that acute exercise activates
369 CREB (49, 142), it is possible that this is responsible, at least in part, for increased SIRT1 gene
370 transcription with exercise. The effects of exercise on ChREBP expression and activation in muscle have
371 not been studied. It is also possible that SIRT1 gene expression is regulated by changes in NADH levels.
372 To this end, SIRT1 gene expression is also regulated by C-terminal binding protein (CtBP) (189), a
373 transcriptional corepressor that has a 100-fold greater affinity for NADH than NAD^+ (53, 188). While we
374 discuss CtBP in more detail later in this review, of note here is that changes in NADH levels during or
375 after exercise could reduce the repressive effects of CtBP on SIRT1 gene transcription in skeletal muscle.

376 ***SIRT3*.** SIRT3 is considered to be a mitochondrial-localized protein (8, 35, 71, 124, 136, 170,
377 172), although there have been some conflicting reports on its localization (166). Relevant to our
378 discussion, in skeletal muscle SIRT3 appears to localize solely to mitochondria (71), and scales with
379 markers of skeletal muscle oxidative capacity (71, 137). Additionally, SIRT3 is decreased in old vs.

young sedentary individuals, but is higher in endurance-trained vs. sedentary individuals, regardless of age (111). In line with this, exercise training or chronic electrical stimulation (71, 82, 137), but not acute exercise (71, 82), increases skeletal muscle SIRT3 protein levels, and is specific to those muscles recruited during the exercise intervention. Complimenting these studies, knockdown of SIRT3 in C2C12 muscle cells decreases basal and maximal oxygen consumption rates and mitochondrial content, and prevents PGC1α-induced activation of mitochondrial genes (86, 105). Although knockdown of SIRT3 does not reduce the total mitochondria number as measured by the abundance of complexes I, III and V of the ETC (86), it does reduce skeletal muscle fatty acid oxidation by ~50%, due to hyperacetylation of long chain acyl CoA dehydrogenase (LCAD) (81). Alternatively, overexpression of SIRT3 in C2C12 myotubes increases mitochondrial DNA content (105). Taken together, these studies suggest that SIRT3 plays an important role in regulating skeletal muscle mitochondrial biogenesis, and potentially fatty acid oxidation, in response to long-term exercise training. However, a recent paper by Yang et al. (183) in C2C12 muscle cells and skeletal muscle from SIRT3 null mice counters this perspective. In their paper, the authors demonstrate that SIRT3 acts to reduce mitochondrial protein synthesis (and thus, mitochondrial biogenesis) via its ability to deacetylate mitochondrial ribosomal protein L10 (MRPL10) and negatively regulate the activity of mitochondrial ribosomes. Thus, rather than increase mitochondrial protein synthesis, SIRT3 appears to have the opposite effect in skeletal muscle. The teleological implications of this will be discussed shortly.

Increased ATP utilization during exercise is matched through increased mitochondrial ATP production, which occurs via oxidation of mitochondrial NADH produced in metabolic pathways such as glycolysis, the TCA cycle, β-oxidation, and the electron transport chain (ETC). Interestingly, up to one fifth of mitochondrial proteins are acetylated, as are many of the proteins in these metabolic pathways, which has important effects on their function (95, 177, 191). Indeed, SIRT3 appears to be responsible for much of the deacetylation of mitochondrial proteins (124, 136, 170, 172). Of potential interest to ATP generation in skeletal muscle during exercise, SIRT3 deacetylates and activates the TCA cycle and ETC

405 enzymes, including succinate dehydrogenase (SDH) (30), ubiquinol-cytochrome c reductase hinge protein
406 (a component of complex III) (114), malate dehydrogenase (137), NDUFA9 of complex I (2), GDH
407 (124), ATP synthase (114), and isocitrate dehydrogenase 2 (ICDH2) (168). Also, SIRT3 deacetylates and
408 activates the β-oxidation enzyme, LCAD (81). With this information in mind, we propose that a possible
409 role of SIRT3 in skeletal muscle is the acute regulation of enzymes and pathways that generate ATP in
410 response to ATP demand during exercise. This is supported by the fact ATP production in heart, kidney,
411 and liver from SIRT3 null mice is reduced by more than 50% (2), although whether this is the case in
412 skeletal muscle is unknown. In the context of the findings of Yang et al. (183) showing that SIRT3
413 reduces (rather than increases) mitochondrial protein synthesis, as measured by a [^{35}S]-methionine
414 translation-based assay, this also would make teleological sense. Thus, during exercise it is necessary to
415 generate ATP to maintain force production, so pathways that utilize energy, such as protein synthesis,
416 would be momentarily halted. The actions of SIRT3, therefore, are akin to the effects of AMPK on
417 enhancing energy production and inhibiting pathways that use energy for processes other than to maintain
418 ATP production and muscle work (79, 85), albeit the effects of SIRT3 are specific to the mitochondria. It
419 will be of interest in future studies to determine if mitochondrial biogenesis in response to exercise is
420 impaired in SIRT3 null mice. Also, given that fatty acid oxidation increases during endurance exercise
421 (38), it will interesting to determine if acute exercise alters substrate utilization in parallel with activation
422 of SIRT3 activity and deacetylation of its downstream targets. Studies using muscle-specific SIRT3 null
423 mice and exercise will no doubt be very informative regarding such questions.
424
425 **PARPs and mitochondrial biogenesis in skeletal muscle**
426 The PARPs are major consumers of nuclear NAD^+, and therefore compete with SIRT1 for NAD^+
427 in the nucleus (40, 76, 174, 185, 193). Considering this, a series of papers from the laboratory of Johan
428 Auwerx recently investigated the effects of knocking down PARP1 and PARP2 on skeletal muscle
429 mitochondrial biogenesis in C2C12 myotubes and mice. PARP1 null mice had increased levels of NAD^+,

reduced acetylation of SIRT1 substrates such as PGC1α and FOXO1, and increased mitochondrial biogenesis, as measured by mitochondrial gene expression, mitochondrial morphology, SDH staining and mtDNA content, O_2 consumption (7). Increased muscle SIRT1 activity may in part be due to increased protein content, although SIRT1 activity was increased in HEK293 cells without an increase in SIRT1 protein content (7). Complimenting these findings, treatment of mice with PARP-1 inhibitors increased NAD^+ levels and SIRT1 activity (7). The activity of other non-nuclear sirtuins including SIRT2 and SIRT3, however, were unchanged in PARP1 null tissues (7), suggesting that the upregulation of SIRT1 in the absence of PARP1 may be due to a local change in the NAD^+ pool in the nuclear compartment. Similar to PARP1, knockdown of PARP2 in C2C12 myocytes increased SIRT1 activity (6). In skeletal muscle this appeared to occur through both an increase in intracellular NAD^+ levels and modulation of the SIRT1 promoter by PARP2 (6). As expected, SIRT1 activity was increased in PARP1 and PARP2 null mice and these mice also had increases in skeletal muscle mitochondrial biogenesis (e.g., mtDNA, mitochondrial morphology and gene expression, SDH staining) and their muscle demonstrated a more oxidative phenotype (6, 7). Moreover, PARP2 null mice had increased endurance as measured by a treadmill endurance test (6). Whether this was due to improvements in skeletal muscle per se, or was a function of the changes in other tissues, such as the heart, was not determined. Collectively these studies are very interesting, and suggest that inhibition of PARPs could be used to enhance muscle mitochondrial biogenesis by increasing nuclear NAD^+ levels and increasing SIRT1 activation. If exercise leads to an increase in NAD^+ in the nuclear compartment, it will be interesting in the future to determine if acute exercise leads to inhibition of PARP1 and PARP2, so as to maximize NAD^+ levels and SIRT1 activation. Although, it is notable that *in vivo* SIRT1 deacetylase activity is not required for the ability of exercise to enhance mitochondrial biogenesis (142). Thus, studies that cross PARP1/2 null mice with mKO^{SIRT1} mice, or studies with PARP inhibitors in mKO^{SIRT1} mice, will help to definitively determine if PARP inhibition works through SIRT1, *in vivo*.

455 **Contribution of NADH to mitochondrial adaptations to exercise: Possible role of CtBP**

456 As discussed above, CtBP is a transcriptional corepressor that is greater than 100-fold more
457 sensitive to perturbations in cellular NADH vs NAD^+ levels (53, 188). Considering that the
458 cytosolic/nuclear content of NAD^+ in muscle is estimated to be ~540-fold higher than NADH (42, 119),
459 conversion of NAD^+ to NADH, or vice versa, would therefore result in a greater change in the NADH
460 levels. By extension, and as reasoned by others (53, 188, 189), changes in nuclear NADH, rather than
461 NAD^+, could link perturbations in NAD^+/NADH ratio to gene transcription. To this end, CtBP regulates
462 mitochondrial morphology and function in MEFs and liver-related cells, via its ability to regulate Bcl-2-
463 associated X protein (Bax) (94). CtBP also represses the transcriptional activity of myocyte enhancer
464 factor 2 (MEF2) (186), a key transcription factor in the regulation of mitochondrial biogenesis (39, 131)
465 that shows increased DNA binding in response to exercise (130). The regulation of MEF2 transcriptional
466 activity, however, is complex, as MEF2 is deacetylated by SIRT1, and deacetylation of MEF2 *in vitro*
467 reduces (not increases) its transcriptional activity (126, 192). So clearly, the interplay of exercise on
468 NAD^+(H), SIRT1, CtBP and MEF2, and the subsequent transcriptional response, may represents a
469 balance of these activating and inhibitory signals, that likely involves additional levels of regulatory
470 control, such as ubiquitination, sumoylation and phosphorylation (67, 92, 149). Taken together, these
471 studies point to a potentially important role of CtBP, via its sensitivity to changes in NADH, in the
472 modulation of mitochondrial biogenesis in skeletal muscle in response to exercise.

473

474 **Replenishing NAD^+ levels in skeletal muscle: An important consideration**

475 If an increase in NAD^+ during exercise leads to an increase in the activity (and thus consumption
476 of NAD^+) by SIRT1, SIRT3, PARP1 or PARP2, then it would be important for skeletal muscle to
477 replenish NAD^+ levels in the cytosolic, nuclear and mitochondrial compartments during or after exercise.
478 In mammals, the NAD^+ biosynthetic and salvage pathways replenish NAD^+, and the specifics of these
479 pathways are reviewed elsewhere (11, 12, 84, 97, 128, 144, 190); an overview of these pathways is

480 presented in Figure 2. Except for research on NAM phosphoribosyltransferase (NAMPT; also known as
481 pre-B-cell colony-enhancing factor 1 (PBEF1) or visfatin), the contribution of these pathways to
482 replenishment of NAD^+ in skeletal muscle and in response to exercise is to date, essentially unstudied.

483 NAMPT is located in the nucleus, cytosol and mitochondria (96, 148, 182), and is part of the
484 NAD^+ biosynthetic pathway that converts NAM to NAM mononucleotide (NMN) (40, 76, 174, 185, 193).
485 This reaction is potentially important for maintaining the activity of SIRT1 and SIRT3, as nicotinamide
486 (which is generated in the deacetylase reaction of sirtuins, including SIRT1 and SIRT3) is a negative
487 regulator of SIRT1 and SIRT3 (11, 12, 84, 97, 128, 144, 169, 190). Indeed, in HEK293 cells, NAMPT
488 plays an important role in protecting against cell death in response to genotoxic stress by maintaining
489 mitochondrial NAD^+ levels and SIRT3 activation (182). However, in plasma from humans and mice
490 NAM concentrations (which range from 0.3 to 5 μM) are lower than the reported IC_{50} for SIRT1
491 inhibition, but is in the range of the K_M for NAMPT (24, 148, 151). Thus, whether or not NAM levels in
492 muscle reach a level sufficient to inhibit SIRT1/SIRT3 is unknown. This aside, in rodents, endurance
493 exercise increases NAMPT gene and/or protein expression in parallel with increased tissue NAD^+ levels
494 (23, 104). Similarly, in humans, NAMPT protein abundance is higher in trained vs. untrained individuals,
495 and is increased by exercise training, although whether this increased NAD^+ levels was not measured
496 (37). Thus, in the context of increased SIRT1/SIRT3 activity during and after exercise, a coordinated
497 increase in NAMPT activity may act to maintain SIRT1/SIRT3 activity by consuming NAM, and also
498 replenishing NAD^+ (discussed below). The concentration of NAM in skeletal muscle is unknown.
499 Therefore, it will be interesting in future studies to determine whether NAMPT activity is increasing
500 specifically in the mitochondrial, nuclear and/or cytosolic compartments with exercise, and whether this
501 coincides with changes in NAM levels. Altogether, such measurements will provide important
502 information regarding the precise contribution of NAMPT to NAD^+ metabolism and the regulation of
503 SIRT1 and SIRT3 activity in skeletal muscle in response to exercise.

To generate NAD^+, NMN generated by the NAMPT reaction is converted by NMN adenylyltransferas (NMNAT) to NAD^+. NMNAT can also convert nicotinic acid (NA) mononucleotide (NAMN) to NA adenine dinucleotide (NAAD), which is subsequently converted to NAD^+, by NAD^+ synthase. There are three isoforms of NMNAT: NMNAT1 and NMNAT2 are localized in the cytosol and nucleus, and NMNAT3 appears to be exclusively in mitochondria (113, 133). At the mRNA level, NMNAT1 is highly expressed in skeletal muscle (51, 52, 113), NMNAT2 is expressed at low levels, whilst NMNAT3 is very low or absent (113). The protein levels and activity of these proteins in skeletal muscle are unknown. The presence of NMNAT1 and to a lower extent, NMNAT2, in skeletal muscle suggests that they may play an important role in replenishing nuclear and cytosolic NAD^+ levels, and it will be interesting to see if exercise coordinately increases NAMPT and NMNAT1/2 levels, in order to maintain the overall cytosolic/nuclear NAD^+ pools. Regarding replenishment of mitochondrial NAD^+, the inner mitochondrial membrane is impermeable to NAD^+ and NADH (115, 143), which poses a potential problem for maintaining the mitochondrial NAD^+ level, particularly if NAD^+ consumption by SIRT3 is increased during exercise. Only recently was it demonstrated in HeLa S3 cells that NMNAT3 is the only known enzyme of NAD^+ synthesis in mitochondria (133). Whilst NMNAT3 gene expression is very low in skeletal muscle, it will be of interest in future studies to determine if NMNAT3 activity in skeletal muscle correlates with mitochondrial density or if exercise increases the activity or abundance of NMNAT3, even independent of an increase in mitochondrial abundance. Alternatively, perhaps a different or an additional mitochondrial NAD^+ salvage or biosynthetic pathway is present in skeletal muscle mitochondria.

Concluding remarks: There are still many unanswered questions

It has been more than 100 years since the discovery of the pyridine nucleotides, NAD^+ and NADH. While for much of this time NAD^+(H) was considered to primarily participate in metabolic reactions that led to generation of ATP through their ability to act as substrates for enzymes or as covalent

529 modifiers of enzyme function, these coenzymes are potentially key mediators of the adaptive response to
530 exercise. Indeed, changes in NAD^+(H) levels in concert with known NAD^+(H) sensing enzymes provides
531 a logical link between exercise-induced metabolic stress and subsequent mitochondrial adaptations.
532 Specifically, the effects of SIRT1, PARP1/2, and CtBP appear to manifest through their ability to directly
533 or indirectly modulate the transcriptional response to exercise; they likely do not contribute to an
534 immediate increase in ATP production during acute exercise (Figure 3). Very little, however, is known
535 about NAD^+(H) dynamics in the nucleus of skeletal muscle, and how this affects the transcriptionally-
536 based adaptations central to endurance exercise training. Regarding SIRT3, we propose that it acts as an
537 acute regulator of mitochondrial ATP production via its ability to regulate the enzymatic activity of
538 various TCA and ETC enzymes (and possible as yet to be discovered targets). An additional component
539 of this acute regulation is proposed to include a reduction in mitochondrial protein synthesis during
540 exercise (Figure 3). It is possible that during exercise, SIRT1 plays a similar role in regulating cellular
541 protein synthesis in the cytosol via its ability to negatively regulate mammalian target of rapamycin
542 (mTOR) and/or its interaction with tuberosclerosis complex 2 (TCS2) (58). Whether this regulation
543 occurs in muscle or during exercise is not known. In addition, little is known regarding the coordination
544 of NAD^+ consuming and regeneration pathways in skeletal muscle and whether these two opposing events
545 are regulated by common mechanisms. Furthermore, our understanding of the compartmentation of
546 NAD^+(H) metabolism, and quantitative changes in NAD^+, NADH, and the NAD^+/NADH ratio in
547 subcellular compartments in skeletal muscle at rest and in response to exercise is poor. While technically
548 challenging to measure, such investigation will be highly informative with respect to understanding the
549 activation or inhibition of both NAD^+(H)-responsive proteins. For example, while NAD^+ can clearly
550 activate sirtuins, NADH can act as a competitive inhibitor of SIRT1 (120). However, the relative binding
551 affinity of NAD^+ for SIRT1, is ~1000-fold greater than NADH, and overall, the ability of NADH to
552 inhibit SIRT1 activity is proposed to be minimal in an *in vivo* setting (169). Thus, determining the precise
553 contribution of changes in NAD^+, NADH, and the NAD^+/NADH ratio will be important. In the end it is

554 likely that a combination of changes in free NAD^+ and NADH levels and the NAD^+/NADH ratio within
555 specific subcellular compartments is important. Thus, as research on NAD^+(H) metabolism continues into
556 its second century, there are still many important research questions to be resolved regarding their effect
557 on the adaptive response to exercise in skeletal muscle. Ultimately, such research holds great promise for
558 improving our fundamental understanding of skeletal muscle function in response to exercise, which has
559 obvious and important implications for human health and treatment of skeletal muscle-related diseases.
560

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Figure Legends

Figure 1. **Compartmentation of NAD^+ and NADH in skeletal muscle.** NAD^+ and NADH move freely across pores in the nuclear membrane, and as such the cytosolic and nuclear compartment concentrations of NAD^+ and NADH are thought to be comparable. In the cytosol, NADH is generated by glycolysis. Because mitochondria are impermeable to NADH, the transfer of these reducing equivalents occurs via a variety of shuttles including the glycerol-3-phosphate shuttle, malate-aspartate shuttle, lactate shuttle, and the NADH/cytochrome c electron transport shuttle, as described in the text. Depending on the shuttle NADH is produced. The cytosolic/nuclear NAD^+ pool is replenished when NADH is converted back to NAD^+ in the reactions of the aforementioned shuttles, including the conversion of pyruvate to lactate. NAD^+ levels in the nuclear, cytosolic, and mitochondrial compartments are also replenished via specific *de novo* and salvage pathways that are discussed in the text and overviewed in Figure 2. Within the mitochondria, NADH is oxidized to NAD^+ in the electron transport chain (ETC).

Figure 2. Replenishment of NAD^+ through the biosynthesis (*de novo*) and salvage pathways. Given there are many NAD^+-consuming enzymes, it is essential that NAD^+ be replenished in order to maintain compartmental NAD^+ levels. This occurs through the salvage and biosynthetic pathways. Except for NAMPT, the role of these pathways in NAD^+ replenishment in skeletal muscle, and in response to exercise, are essentially unknown. Molecules generated in each pathway are in orange. Enzymes are in blue. NA, nicotinic acid; NAM, nicotinamide; NAMN, NA mononucleotide; NMN, NAM mononucleotide; NMNAT, NMN adenylyltransferase; NAAD, NA adenine dinucleotide; NAD^+, NAM adenine dinucleotide; NAPT, NA phosphoribosyltransferase; NAMPT, NAM phosphoribosyltransferase; NR, nicotinamide riboside.

Figure 3. Proposed mechanism for exercise-induced mitochondrial biogenesis via NAD^+/NADH metabolism. Increased ATP demand during exercise leads to an increase in the free cytosolic/nuclear and

mitochondrial NAD^+ level and NAD^+/NADH ratio, which provides increased substrate for the NAD^+-consuming enzymes (in purple), SIRT1, SIRT3, PARP1 and PARP2. Exercise also reduces the availability of NADH, the predominant covalent activator of CtBP. It is hypothesized that during exercise, increased ATP production is facilitated by SIRT3-mediated deacetylation of a series of enzymes in the TCA, β-oxidation and ETC. In parallel, SIRT3 acutely reduces mitochondrial protein synthesis, which maximizes the availability of reducing equivalents for ATP production. Whether SIRT3 is required for induction of mitochondrial biogenesis after exercise remains to be determined. In response to exercise SIRT1 is also activated by increased cytosolic/nuclear NAD^+ levels, and while it likely can contribute to mitochondrial biogenesis through PGC1α-dependent and -independent mechanisms, it is not required for exercise-mediated deacetylation of PGC1α. Rather, acute exercise appears to reduce the inhibitory effect of the acetyltransferase, GCN5, on PGC1α, via mechanism that is still to be determined. PARP1 and PARP2 are able to directly or indirectly modulate SIRT1 activity (and mitochondrial biogenesis) by competing for NAD^+, although the effects of exercise on the activity of these enzymes is unknown. Also, whether SIRT1 is required for the ability of PARP inhibition to induce mitochondrial biogenesis in skeletal muscle, *in vivo*, is not known. The transcriptional corepressor CtBP, is activated by NADH, and it is hypothesized that during or after exercise that reductions in the cytosolic/nuclear NADH level reduces the repressive effects of CtBP on transcriptional modulators of mitochondrial biogenesis. Ultimately, increased activity of enzymes of the ETC, TCA cycle and β-oxidation, and/or increased mitochondrial number (i.e., biogenesis) leads to an enhanced capacity of the mitochondria and muscle to generate ATP. Legend: Dotted lines indicate that a hypothesized contribution of the pathway, or that the data to date provides an incomplete perspective. PARP, poly [ADP-ribose] polymerase; NAD^+, nicotinamide adenine nucleotide (oxidized); NADH, nicotinamide adenine nucleotide (reduced); SIRT1, sirtuin 1; SIRT3, sirtuin 3; GCN5, general control of amino acid synthesis; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; CtBP, C-terminal binding protein.

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Figure 1

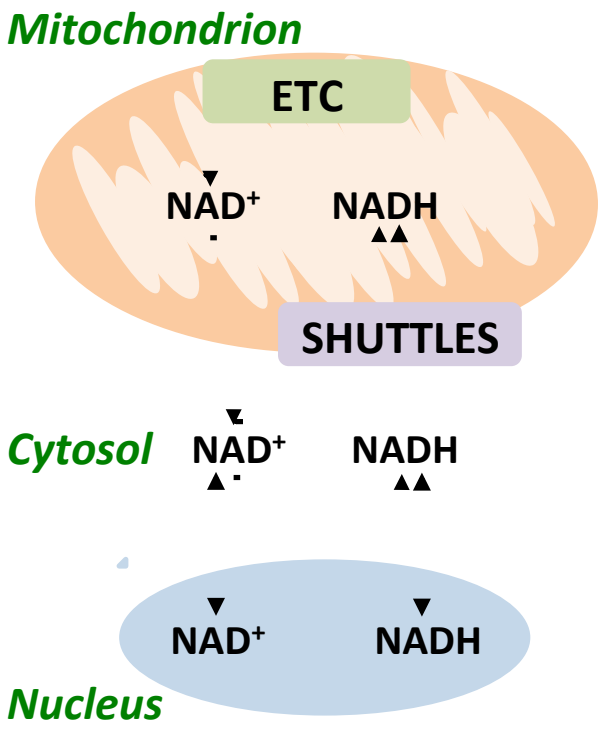

Mitochondrion
ETC
NAD+
NADH
SHUTTLES
Cytosol
NAD+
NADH
NAD+
NADH
Nucleus

Figure 2

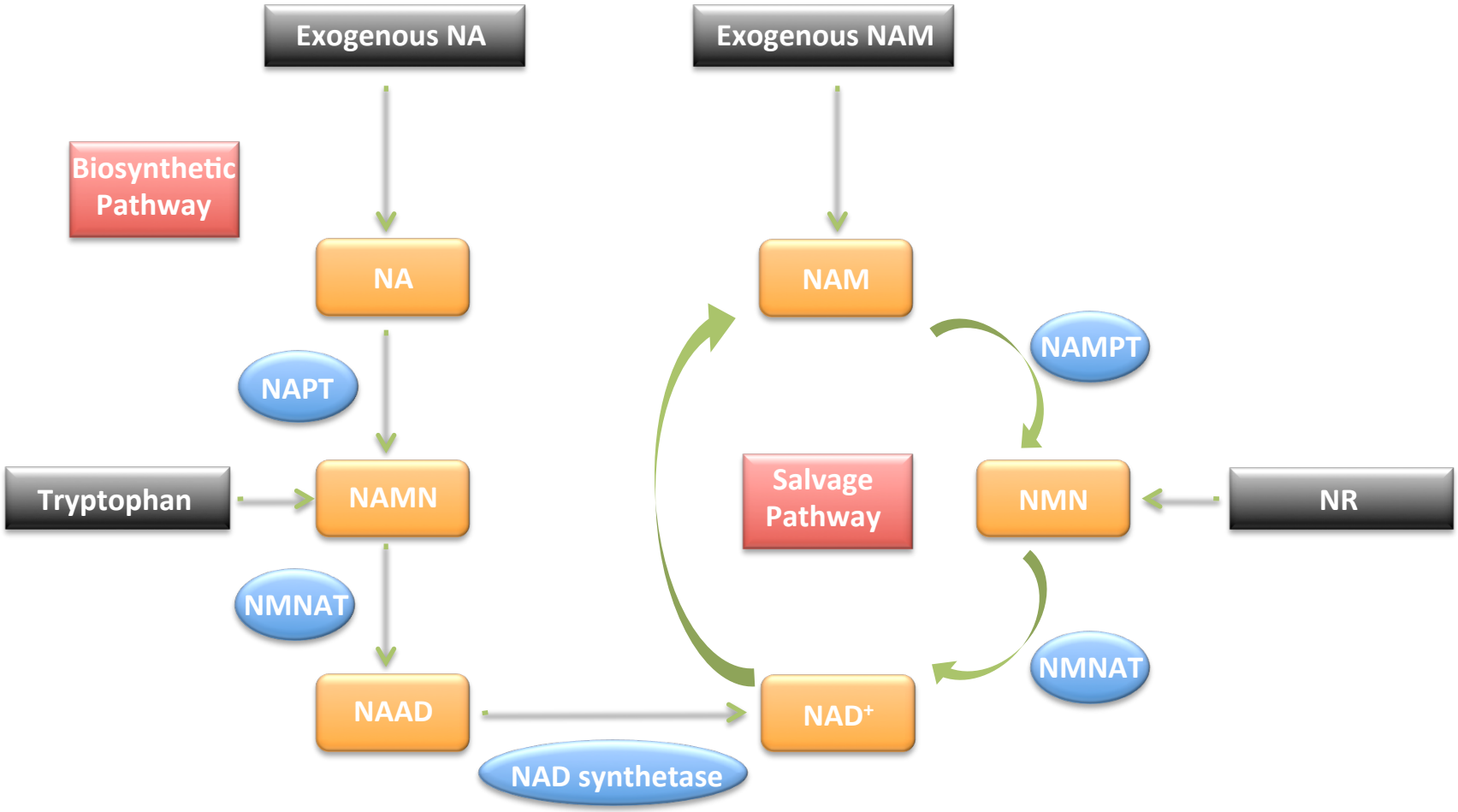
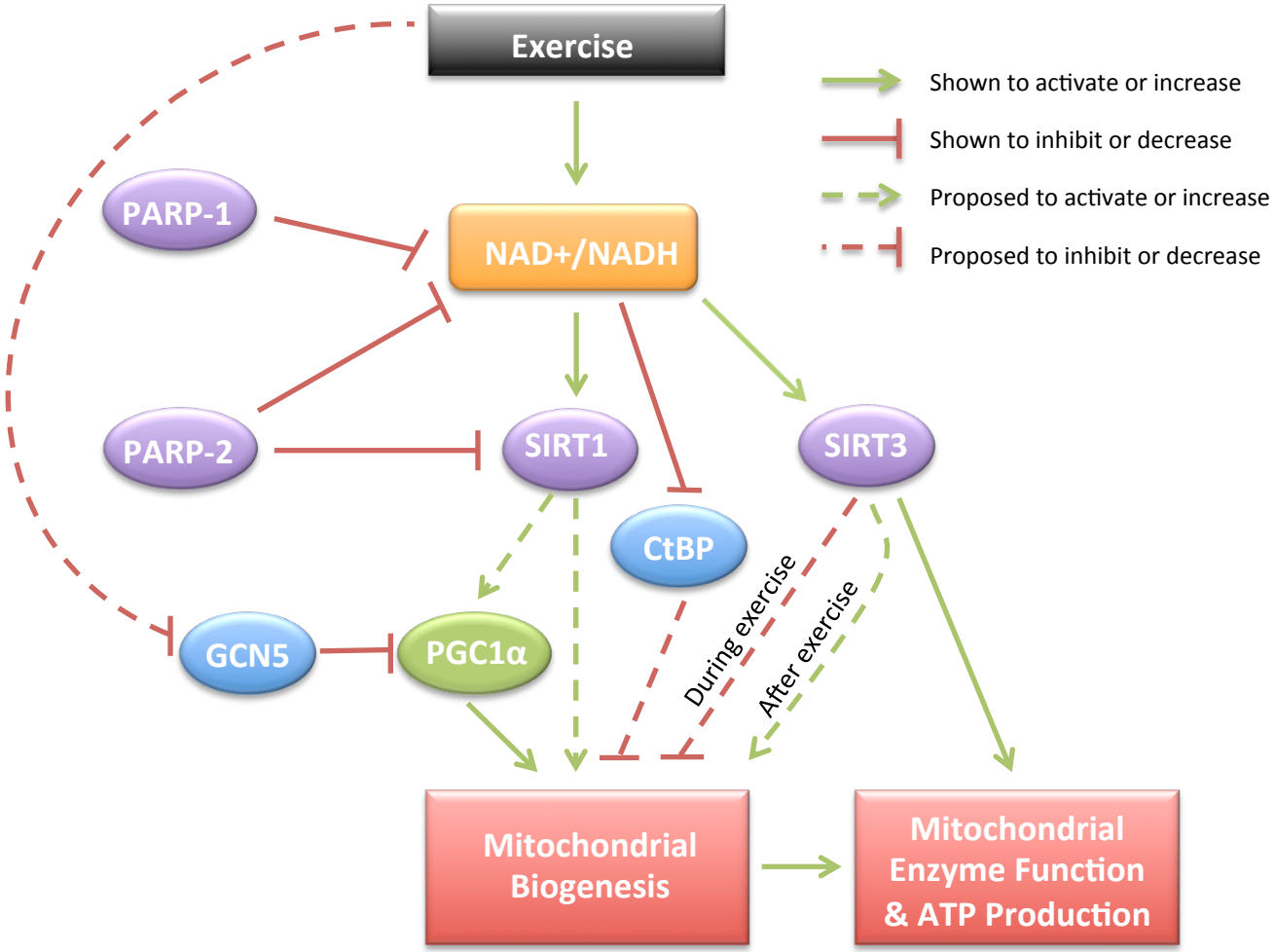

Exogenous NA
Exogenous NAM
Biosynthetic Pathway
NA
NAM
NAPT
NAMPT
Tryptophan
NAMN
Salvage Pathway
NMN
NR
NMNAT
NMNAT
NAAD
NAD+
NAD synthetase

Figure 3


Exercise
PARP-1
NAD+/NADH
PARP-2
SIRT1
SIRT3
CtBP
GCN5
PGC1α
During exercise
After exercise
Mitochondrial Biogenesis
Mitochondrial Enzyme Function & ATP Production
Shown to activate or increase
Shown to inhibit or decrease
Proposed to activate or increase
Proposed to inhibit or decrease