Acute sources of mitochondrial NAD\(^+\) during respiratory chain dysfunction

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**ABSTRACT**

It is a textbook definition that in the absence of oxygen or inhibition of the mitochondrial respiratory chain by pharmacologic or genetic means, hyper-reduction of the matrix pyridine nucleotide pool ensues due to impairment of complex I oxidizing NADH, leading to reductive stress. However, even under these conditions, the ketoglutarate dehydrogenase complex (KGDHC) is known to provide succinyl-CoA to succinyl-CoA ligase, thus supporting mitochondrial substrate-level phosphorylation (mSLP). Mindful that KGDHC is dependent on provision of NAD\(^+\), hereby sources of acute NADH oxidation are reviewed, namely i) mitochondrial diaphorases, ii) reversal of mitochondrial malate dehydrogenase, iii) reversal of the mitochondrial isocitrate dehydrogenase as it occurs under acidic conditions, iv) residual complex I activity and v) reverse operation of the malate-aspartate shuttle. The concept of NAD\(^+\) import through the inner mitochondrial membrane as well as artificial means of manipulating matrix NAD\(^+\)/NADH are also discussed. Understanding the above mechanisms providing NAD\(^+\) to KGDHC thus supporting mSLP may assist in dampening mitochondrial dysfunction underlying neurological disorders encompassing impairment of the electron transport chain.

1. **Introduction: NAD\(^+\)**

Nicotinamide adenine dinucleotide (NAD\(^+\)) was discovered by Harden and Young in 1906 as a low molecular weight substance present in yeast extract stimulating fermentation (Harden and Young, 1906). Since then and through more than 80,000 publications, NAD\(^+\) has been identified as a reactant in hundreds of reactions, a redox cofactor and a key signaling molecule regulating the cell’s response to environmental changes (Rajman et al., 2018), (Xiao et al., 2018), (Katsyuba et al., 2020). According to BRENDA database (Jeske et al., 2019), an electronic resource comprising extensive information on IUBMB-classified enzymes [https://www.brenda-enzymes.org/], NAD\(^+\) is a substrate or product in 633 reactions occurring in the human body; however, this is likely an overestimation, because by combining several databases and applying sophisticated bioinformatic analysis the group of Mootha reported that in the liver there are 352 known or predicted enzymes producing or consuming NADP(H) or NAD(H) or using them as a redox co-factor (Goodman et al., 2018).

A mammalian cell is estimated to contain 0.2–0.5 mM [NAD\(^+\)] \((Canto et al., 2015), (Yang et al., 2007)\), although it cannot be overly emphasized that exactly due to its participation in many processes its levels vary considerably in response to diverse stimuli involving nutritional challenges, exercise and circadian cycles (Canto et al., 2015), (Goodman et al., 2018). Adding to this complexity is the distribution of NAD\(^+\) among subcellular compartments; allowing for cell-to-cell variations, cytosolic/nuclear NAD\(^+\) is ~100 μM, while mitochondria contain ~250 μM (Cambronne et al., 2016). Furthermore -under normal conditions- in the cytosol the cytosol/NADH/NAD\(^+\) can be up to 1:1000 (Veech et al., 1969). On the other hand, in mitochondria the NADH/NAD\(^+\) ratio is 1:10 due to a more reduced matrix environment, depending on respiratory and metabolic activity (Veech et al., 1969). For excellent, most recent reviews regarding NAD\(^+\) compartmentation the reader is referred to that by (Kulkarni and Brookes, 2019) and (Katsyuba et al., 2020). Finally, it is important to consider that a fraction of total [NAD\(^+\)] is bound to proteins, thus it cannot contribute to the free NAD\(^+\)/NADH ratio (Ansari and Raghava, 2010).

2. **NAD\(^+\) involvement in neurological diseases**

Mindful that NAD\(^+\) exhibits a critical role in diverse cellular processes (Garten et al., 2015) and in view of the plethora of findings soon after the discovery that it is a co-substrate for sirtuins and poly-ADP-ribose polymerases (PARPs) (Imai and Guarente, 2014), (Gibson and Kraus, 2012), realizing its involvement in human disease was inevitable. This unfolds through many paths, the most well-studied being PARP hyper-activation leading to severe depletion of cellular NAD\(^+\) stores in response to extensive DNA damage (Bai, 2015), (Belenky et al., 2007), (Morales et al., 2014).

Involvement of NAD\(^+\) in disorders of the nervous system is no exception (Owens et al., 2013), (Imai and Guarente, 2014), (Katsyuba and...
Auwerx, 2017), (Katsyuba et al., 2020); there even more so, a mitochondrial protein, SARM1, was reported to cause cell destruction through depletion of NAD⁺ in neuronal injuries (Essuman et al., 2017), (Gerds et al., 2015), (Osterloh et al., 2012), thus pinpointing the enzyme as a promising therapeutic drug target (Conforti et al., 2014), (Gerds et al., 2018). Apart from SARM1, mitochondria also harbor Nudix hydrolases, a superfamily of hydrolytic ‘housecleaning’ enzymes that catalyze the cleavage of nucleoside diphosphates linked to x (i.e., any moiety) (Bessman et al., 1996), (McLennan, 2006), (see (Shumar et al., 2018) for an exception to this rule). A link between mitochondrial Nudix hydrolases and neurodegeneration has been addressed (Long et al., 2020).

From the above it is evident that since the deficiency of NAD⁺ may lead to neuropathology, elevating its concentration has been shown to be neuroprotective in many settings, such as in neuronal death induced by ischemic brain damage (Klaidman et al., 2003), (Sadana-Akiyoshi et al., 2003), (Kabra et al., 2004), (Feng et al., 2006), (Kaundal et al., 2003), (Xie et al., 2017), (Zheng et al., 2012), axonal degeneration in spinal cord injury (Xie et al., 2017), traumatic brain injury, multiple sclerosis, Alzheimer’s disease, Parkinson’s disease (Lingor et al., 2012), (Johnson et al., 2013), (Wang et al., 2005), (Sasaki et al., 2006), (Araki et al., 2004) or thalidomide (Qin et al., 2006), (Gong et al., 2013), (Liu et al., 2004), (Turunc Bayrakdar et al., 2014), (Wang et al., 2016), or thwarted degenerative progress of both neurons (Lehmann et al., 2017) (Zhou et al., 2015), (Hampy et al., 2017) and astrocytes (Alano et al., 2004). Neuroprotective effects of elevating intracellular NAD⁺ was also reported in ophthalmic and auditory degeneration models (Shindler et al., 2007), (Williams et al., 2017), (Brown et al., 2014), (Somesy et al., 2010). It is thus not surprising that big pharma expresses intense interest in regimes boosting intracellular NAD⁺ levels (Yoshino et al., 2018), (Bogdan and Brenner, 2008), (Katsyuba et al., 2020).

Having established that intracellular NAD⁺ deficiency may lead to neuropathology, it remains to be understood as of why is this occurring. Although this is not the topic of the present review, it is worth mentioning that despite that a decline in electron transport chain (ETC) activity associated with human diseases (Vafai and Mootha, 2012), (Wallace, 2005) has been traditionally attributed to a diminished capacity for ATP production through OXPHOS, the contribution of the associated reduction in NADH/NAD⁺ (i.e. decrease in [NAD⁺]) has not been addressed adequately (Vafai and Mootha, 2012). This decrease in NADH/NAD⁺ ratio commonly referred to as “reductive stress” (Xiao and Loscalzo, 2019) forms a rapidly developing emerging topic of interest, see under section “reductive stress”; the vast body of reviews have focused on rather chronic, more sustained NAD⁺-boosting strategies (Canto et al., 2015), (Klimova and Kristian, 2019), (Katsyuba et al., 2020) and references therein. Hereby, multiple sources of NADH oxidation in the matrix are being discussed, which could be sources of immediate provision for NAD⁺.

3. Reductive stress

“Reductive stress” was a term introduced by the group of Lemasters in 1989 while demonstrating that in rat hepatocytes undergoing chemical anoxia, a blockade in mitochondrial respiration and ATP production ensued (Gores et al., 1989). The authors proposed that upon reoxidation, the electron carriers which were over-reduced during the hypoxia treatment led to a burst of ROS generation which they termed “reductive stress”. Currently, a more generalized definition is applied in which an imbalance between cellular pro-oxidant levels and reducing capacity in favor of the latter is in place (Handy and Loscalzo, 2012), (Loscalzo, 2016), (Sarsour et al., 2009). In the vast majority of cases, reductive stress refers to an excess of NADH, NADPH, GSH and protein cysteine thiols over the respective oxidized counterparts (Xiao and Loscalzo, 2019), (Perez-Torres et al., 2017). This reductive stress by means of diminishing cell growth responses altering the balance of protein disulfide bonds in proteins, reducing mitochondrial functions and decreasing cellular metabolism (Perez-Torres et al., 2017), (Maity et al., 2016), (Xiao and Loscalzo, 2019) contribute to the development of many diverse diseases (Handy and Loscalzo, 2017), (Perez-Torres et al., 2017), (Xiao and Loscalzo, 2019), including those encompassing neurodegeneration (Lloret et al., 2016), (Wu et al., 2016). Mechanisms integrating reductive stress and diverse conditions are summarized in Fig. 1 (obtained from (Perez-Torres et al., 2017) distributed under the Creative Commons Attribution License v 4.0). Finally, the notion that addition of pyruvate to Rhö0 cells which are respiration-deficient due to deleterious mtDNA mutations (or complete lack thereof) (King and
4. Acute provision of NAD$^+$ through mitochondrial diaphorases

Diaphorases are flavoenzymes catalyzing the oxidation of NAD(P)H by endogenous or artificial electron acceptors. Purification of a diaphorase was first reported by Bruno Ferenc Straub in 1939 (Straub, 1939). After the hiatus in research due to the II World War, research on diaphorases was spearheaded by Lars Ernster; he coined the term DT-diaphorases because of their reactivity with both DPNH (NADH) and TPNH (NADPH). Unfortunately, many articles authored by Lars Ernster and colleagues are not indexed in PUBMED, however, those published in Acta Chemica Scandinavica since 1947 are available in this link http://actachemscand.org. Almost simultaneously, the enzyme has been identified by Märki and Martius (Märki and Martius, 1961) which they termed vitamin K reductase (Ernster et al., 1962). Quinone reductases with properties similar to that described by Ernster have been reported earlier by Wosilait and colleagues (Wosilait and Nason, 1954), (Wosilait et al., 1954), Williams and colleagues (Williams Jr. et al., 1959), Giuditta and Streeker (Giuditta and Streeker, 1959), (Giuditta and Streeker, 1961) and Koli and colleagues (Koli et al., 1969). From the mechanistic point of view, a DT-diaphorase (EC 1.6.5.2, formerly assigned to EC 1.6.99.2) catalyzes the 2-electron reductive metabolism oxidizing NAD(P)H, thus providing NAD(P)$^+$, while reducing suitable quinones. Research on diaphorases was intense until the 60’s as it was believed them to be an important part of energy-harnessing in mitochondria, but the field became essentially dormant when Mitchell’s chemiosmotic theory was universally accepted. Several isoforms have been identified (Long and Jaiswal, 2000), (Vasiliou et al., 2004); among them, NQO1 and NQO2 have been most extensively characterized (Long and Jaiswal, 2000). A striking difference among them is that NQO2 uses dihydrodiconitinamide riboside (NRH), while NQO1 uses NAD(P)H as an electron donor (Wu et al., 1997), (Zhao et al., 1997). NQO1 is distributed in the cytosol and mitochondria (Ernster et al., 1962), (Dong et al., 2013), (Bianchet et al., 2013), (Elässon et al., 1999), (Edlund et al., 1982), (Conover and Ernster, 1962), (Conover and Ernster, 1960), (Wosilait, 1960), (Colpa-Boonstra and Slater, 1958), (Lind and Hojeberg, 1981), but see (Winski et al., 2002). Total mitochondrial diaphorase activity corresponds to 3–15% of total cellular activity (Ernster et al., 1962), (Edlund et al., 1982), (Wosilait, 1960), (Colpa-Boonstra and Slater, 1958), (Lind and Hojeberg, 1981) and is localized in the matrix, since it reacts only with intramitochondrial reduced pyridine nucleotides, but is inaccessible to those added from the outside (Conover and Ernster, 1960), (Conover and Ernster, 1963). Several other mitochondrial enzymes may exhibit diaphorase-like activity, such as the DLD subunit of KGDHC (Massey, 1960), (Klyachko et al., 2005), (Reed and Oliver, 1968), (Ide et al., 1967), (Bando and Aki, 1992), (Vaubel et al., 2011).

In 2014 we reported that mitochondrial diaphorases in the mouse liver contribute up to 81% to the NAD$^+$ pool during respiratory inhibition (Kiss et al., 2014). This was sufficient to maintain operation of KGDHC, which is essential for provision of succinyl-CoA to succinyl-CoA ligase manifesting in the forward operation of adenine nucleotide translocase, thus supporting mSLP, see Fig. 2. No such phenomena were observed in mitochondria obtained from pigeon liver, where DT-diaphorases are known to be absent (Kiss et al., 2014), and references therein. Relevant to this, it is noteworthy that 1–4% of the human population exhibit a polymorphic version of NQO1; tissues from these individuals do not exhibit NAD(P)H: quinone oxidoreductase activity (Traver et al., 1997). Furthermore, we also showed that quinone reoxidation (for the diaphorases) was mediated by complex III; this is in line with the first reports by Conover and Ernster noting that electrons provided by diaphorase substrates enter the electron transport chain at the level of cytochrome b of complex III (Conover and Ernster, 1962). In accordance to this, by studying cyanide-resistant respiration and using artificial acceptors in isolated mitochondria, the group of Iaguzhinskii reported the stimulatory effect of various diaphorase substrates (Kolesova et al., 1991), (Kolesova et al., 1993), (Kolesova et al., 1987), (Kolesova et al., 1989). Consistent with this, menadione conferred protection in an ischemia model, and this was abolished by the complex III inhibitor myxothiazol (Yue et al., 2001), in agreement with the finding that menadione supports mitochondrial respiration with an inhibited complex I but not complex III (Conover and Ernster, 1962). Also, the cytotoxicity caused by complex I inhibitor rotenone, but not that caused by complex III inhibitor antimycin, was prevented by CoQ1 or menadione (Chan et al., 2002). Similarly, in HepG2 cells, lymphocytes and primary hepatocytes, both idebenone and CoQ1, but not CoQ10, partially restored cellular ATP levels under conditions of impaired complex I function, in an antimycin-sensitive manner (Haeffeli et al., 2011), (Chan et al., 2002), (Dedukhova et al., 1986). More recently, in 2018 we reported that 2-methoxy-1,4-naptoquinone (MNQ) is preferentially reduced by matrix Nqo1 yielding NAD$^+$ to KGDHC (Ravasz et al., 2018). Collectively, our results implied that the re-oxidation of substrates being used by the diaphorases for generation of NAD$^+$ during respiratory arrest by rotenone is mediated by complex III. This protective mechanism by matrix diaphorases comes into play when ETC is inhibited by complex I inhibitors and not in the presence of anoxia, because in the latter case electron acceptors for complex III are not available. However, p66Shc, a protein residing in the intermembrane space of mitochondria (Vasiliou et al., 2004), (Giorgio et al., 2005), is known to oxidize cytochrome c (Giorgio et al., 2005) and it could be possible that oxygen unavailability may not hinder the protective function of diaphorases. Even oxidized glutathione has been reported to reduce cytochrome c (Ames and Elvehjem, 1946), (Painter and Hunter Jr., 1970). Reduction of cytochrome c in vitro by mitochondrial thioredoxin reductase (TrxR2) using NADH has also been reported (Navarrete et al., 2004). Ascorbate is also a potentially suitable oxidizing agent that can re-oxidize cytochrome c, and it is well-known that neurons may harbor large amounts of ascorbate (Grunevald, 1993). Overall, provision of suitable quinones to matrix diaphorases may ensure adequate NAD$^+$ for KGDHC, when the electron transport chain is inhibited. A considerable amount of published data exists linking diaphorases to neurodegenerative diseases, but they are evaluated exclusively from the point of view that diaphorases act upon redox-active substances, thus it will not be reviewed.

5. Acute provision of NAD$^+$ through reversal of mitochondrial malate dehydrogenase

Mitochondria harbor an NAD$^+$-dependent malate dehydrogenase encoded by MDH2 catalyzing the reaction: malate + NAD$^+$ < - > oxaloacetate + NADH. MDH2 is strongly favored towards reduction of oxaloacetate due to a large positive change of $\Delta$G (+28.04 kJ/mol) (Chinopoulos, 2013). However, in mitochondria, it proceeds towards oxaloacetate because the larger $\Delta$G in the negative range (-36.6 kJ/mol) (Chinopoulos, 2013) of citrate synthase pulls the reaction along and keeps oxaloacetate concentration at a very low level. MDH2 is also subject to regulation by several metabolites: the enzyme is activated by citrate in the NAD$^+$ -> NADH direction and inhibited by citrate in the NADH -> NAD$^+$ direction (Mullinax et al., 1982), (Havranova et al., 1979). Regulation of MDH2 by citrate may be of physiological significance (McEvily et al., 1985), as the citrate dissociation constant is ~2 mM, thus within the physiological range concentration (Siess et al., 1977), (Tischler et al., 1977), (Watkins et al., 1977). The potential physiological role of MDH2 regulation is further supported by the finding that citrate synthase and MDH2 form a complex (Fahien and Kmito´ek, 1983) while oxaloacetate controls citrate synthase activity (Kreb, 1970). Oxaloacetate also controls MDH2 activity (Kaval and
MDH2 can be regulated to operate towards NADH oxidation, see Fig. 2; specifically, MDH2 activity is much higher than that of the remaining enzymes, notably KGDHC. Thus, even a mild activity of MDH2 operating in reverse may suffice to have MDH2 reversal in full: in the human brain during anoxia, α-ketoglutarate is oxidized to succinate and CO₂, while oxaloacetate is reduced to malate. This practically means that α-ketoglutarate was being transformed to succinyl-CoA using NAD⁺ coming from mitochondrial malate dehydrogenase reversal. It is not necessary to have MDH2 reversal in full: in the human brain as well as other tissues, MDH2 activity is much higher than that of the remaining enzymes of the citric acid cycle (Bubber et al., 2005), see Fig. 3; more specifically, MDH2 exhibits a ~40 times higher activity compared to that of KGDHC. Thus, even a mild activity of MDH2 operating in reverse may suffice for yielding NAD⁺ for KGDHC. The availability of cells from patients suffering from MDH2 deficiency (Ait-El-Mkadem et al., 2017), as well as the recently described specific MDH2 inhibitors (Ban et al., 2016), (Lee et al., 2013), (Naik et al., 2014) may provide valuable tools in deciphering the potential contribution of MDH2 reversal providing NAD⁺ in the matrix of anoxic mitochondria. Finally, it may be of value to consider that MDH2 activity is elevated in brains of patients with Alzheimer’s disease (Bubber et al., 2005), (Op den Velde and Stam, 1976), (Shi and Gibson, 2011), perhaps serving the purpose of alleviating a decrease in matrix [NAD⁺]. One potential issue arising though from the assertion that reverse MDH2 flux could be a source of NAD⁺ is that this would lead to a build-up in fumarate concentration in the matrix, especially in view of the fact that SDH directionality also remains towards production of fumarate. Several mechanisms are in place that could compensate this build-up: i) both succinate and fumarate may exit mitochondria and this is indeed what sustains the “hypoxic response” mediated through HIF-1α (Benit et al., 2014), (Raimundo et al., 2011), (Semenza, 2007), (Guillemin and Krasnow, 1997); ii) in humans, fumarate is a substrate for three reactions, dihydroporotate dehydrogenase, (EC 1.3.98.1), fumarate hydratase (EC 4.2.1.2) and argininosuccinate lyase (EC 4.3.2.1); iii) fumarate is also a product in four other reactions some of which could operate in reverse diminishing fumarate concentration, namely fumarlyacetocoease (EC 3.7.1.2), acylpyruvate hydratase (EC 3.7.1.5), oxaloacetate decarboxylase (EC 4.1.1.112) and adenylosuccinate lyase (EC 4.3.2.2). Reverse SDH operation diminishing fumarate concentration does not occur to an appreciable extent, for the reasons outlined in (Chinopoulos, 2019). Thus, a potential fumarate build-up due to reverse MDH2 operation in concert with fumarate hydratase reaction can be alleviated via multiple ways.

Fig. 2. Reactions oxidizing NADH in the matrix during mitochondrial respiratory arrest. α-Kg: α-ketoglutarate; c I: complex I of the respiratory chain; c III: complex III of the respiratory chain; c IV: complex IV of the respiratory chain; FH: fumarate hydratase; GLUD: glutamate dehydrogenase; GOT1: aspartate aminotransferase isofrom 1 (cytosolic) GOT2: aspartate aminotransferase isofrom 2 (mitochondrial); KGDHC: α-ketoglutarate dehydrogenase complex; MDH1: malate dehydrogenase isofrom 1 (cytosolic); MDH2: malate dehydrogenase isofrom 2 (mitochondrial); SDH: succinate dehydrogenase; SUCL: succinate-CoA ligase; UQ: Ubiquinone; UQH2: Ubiquinol. The entity transporting NAD⁺ across the inner mitochondrial membrane remains to be identified.

6. Acute provision of NAD⁺ through reversal of isocitrate dehydrogenase

There are 3 isoforms of isocitrate dehydrogenase: A cytosolic NADP⁺-dependent (IDH1), a mitochondrial NAD⁺-dependent (IDH2), and a mitochondrial NAD⁺-dependent (IDH3). Under acidic conditions, IDH2 has been documented to operate in reverse towards formation of NAD⁺, reductively carboxylating α-ketoglutarate to isocitrate (Wise et al., 2011), (Nadtochiy et al., 2016). Relevant to this, it is important to emphasize that acidic pH is a hallmark of ischemia/hypoxia (Rouslin and Broge, 1989), (Katsuura et al., 1991). It can be envisaged that in concert with NAD(P) transhydrogenase encoded by NNT gene, a reverse-operating IDH2 could generate NAD⁺. However, succinate generation in ischemia in a setting that depends on matrix NAD⁺ provision (see under “acute provision of NAD⁺ through residual complex I activity” in tissues obtained from either C57BL6N or C57BL6J mice were similar; this is surprising, because the C57BL/6J mouse strain (from Jax labs) exhibits a polymorphism in the NNT gene, while the C57BL/6N mouse (from Ev Taconic) does not have this mutation. Thus, NNT may not have a role in NAD⁺ formation through IDH2 reversal (Brookes PS, personal communication). The sole role of NNT has been addressed in
(Kiss et al., 2014) and no evidence as matrix NAD⁺ donor was found at least in these settings (inhibited respiratory chain, isolated mouse liver mitochondria), while NNT is known to play an immense role regarding NAD(P)⁺ provision in other experimental models, reviewed in (Xiao and Loscalzo, 2019). Regarding the mitochondrial NAD⁺-dependent IDH3, the possibility of this enzyme operating in reverse potentially providing NAD⁺ has never been thoroughly examined and at this stage it cannot be excluded. Activity of KGDHC (oxidative decarboxylation of α-ketoglutarate) and reverse operation of IDH3 and perhaps IDH2 (reductive carboxylation of α-ketoglutarate) may occur simultaneously without depleting the matrix from α-ketoglutarate; this is because the latter metabolite originates from either glutamate dehydrogenase and/or aspartate aminotransferase, two enzymes with much higher specific activities than both KGDHC and IDH2/3 (Plaitakis and Zaganas, 2001), (McKenna, 2011). This is also why it is meaningful that glutamate dehydrogenase is subject to complex allosteric regulation by several metabolites, controlling the flux of glutamate oxidation in the citric acid cycle (Plaitakis and Zaganas, 2001).

7. Acute provision of NAD⁺ through residual complex I activity

Complex I is instrumental in maintaining NADH oxidation and ubiquinone reduction allowing oxidative phosphorylation, provided that oxygen is available and no other impediments within the respiratory chain are present; however, it must be emphasized that its deficiency does not lead to a lethal phenotype: this is best exemplified by the fact that complex I-associated pathology spans a variety of disorders, i.e. it is not immediately incompatible with life (Abramov and Angelova, 2019). However, when this complex is intact but its operation is hindered by downstream blockade of the respiratory chain as it occurs in hypoxia, residual activity may play a critical role in maintaining sufficient NADH oxidation for supporting KGDHC with NAD⁺ in the citric acid cycle. Indeed, in 1967, Hoberman and Prosky reported that inclusion of rotenone in the Ringer’s liver perfusate with a limited O₂ tension mimicking incomplete anaerobiosis yielded less succinate than in the absence of this complex I inhibitor (Hoberman and Prosky, 1967). In the same line of thought, the group of Brookes showed that during anoxia, rotenone also led to a decrease in succinate production in primary cardiomyocytes (Zhang et al., 2018). Mindful that in anoxia, succinate originates mostly from the canonical activity of the citric acid cycle and not through reversal of succinate dehydrogenase (Zhang et al., 2018), (Chinopoulos, 2019), it may well be possible that complex I exhibits sufficient residual activity for oxidizing NADH yielding NAD⁺ that can support KGDHC activity and ultimately succinate production though mSLP. This is not far-fetched, considering the model published by Jin and Bethke (Jin and Bethke, 2002): The rate equation for complex I activity (J_C1) can be formulated as:

\[ J_{C1} = V_{max} \times \frac{[NADH]/[NAD^+]}{[Q]/[QH_2]} \text{total pool} \times \frac{F_T}{2} \text{where } F_T \text{is thermodynamic drive. All concentrations are in matrix, } [NAD]/[NADH] = [NAD^+] + [NADH]; [Q]/[QH_2] = [Q] + [QH_2]. \]

Acknowledging that in the reaction catalyzed by complex I one molecule of NADH is oxidized to NAD⁺, one molecule of Q is reduced to QH₂ and 4 protons are pumped out of the matrix, complex I activity can be expressed as a function of QH₂/Q and NAD⁺/NADH. The redox ratio of CoQ9 and CoQ10 (QH₂/Q) varies from 0.1 to 100 (Turunen et al., 2004), (Gal nier et al., 2004), in plasma ~20 (Yamamoto and Yamashita, 1997), and in submitochondrial particles 0.1–5 (Kroger and Klingenberg, 1973), while matrix NAD⁺/NADH fluctuates between 0.1 and 10 (Kulkarni and Brookes, 2019). As shown in Fig. 4, the blue area of the 3D plot represents complex I activity (expressed in nmol/e⁻/min/mg) during a wide range of QH₂/Q and NAD⁺/NADH values. In the red area, complex I activity is outlined during anoxic conditions, i.e. when QH₂/Q is expected to be very high, and NAD⁺/NADH very low. It is immediately evident that even under these conditions complex I may retain 9–25% of its total activity to oxidize NADH. From this mathematical modeling it can be inferred that complex I does retain a residual

Fig. 3. Activities of tricarboxylic acid cycle in brains from humans and mice. Note the differences in the vertical axis. The values for humans are the means ± SEM of 13 brains. The values for mice are the means of ± SEM of four mice. PDHC = pyruvate dehydrogenase complex; ICDH = isocitrate dehydrogenase; KGDHC = α-ketoglutarate dehydrogenase complex; STH = succinate thiokinase; SDH = succinate dehydrogenase; CS = citrate synthase; MDH = malate dehydrogenase.
activity even during anoxic conditions, thus exhibiting the capacity for NADH oxidation to the extent of yielding NAD\(^+\) for KGDHC.

Having said that, it is important to emphasize that the decrease in NAD\(^+\)/NADH observed by NADH autofluorescence (Scholz et al., 1995) during anoxia may be overestimated. This is because the binding of NADH to complex I enhances its fluorescence (Blinova et al., 2008) leading to the erroneous impression of an increase in [NADH]. Recent technological advances (Blacker et al., 2014), may provide more accurate measurements regarding matrix NAD\(^+\)/NADH of mitochondria with a dysfunctional respiratory chain (Blacker and Duchen, 2016).

8. Acute provision of NAD\(^+\) through reverse-operating malate-aspartate shuttle

Apart from residual activity of complex I and the aforementioned mechanisms, other sources of NADH oxidation may be present: indeed it was shown that inhibition of complex I (but not complex III) allowed malate-aspartate shuttle (MAS) to operate even during anoxic conditions, thus exhibiting the capacity for NADH oxidation to the extent of yielding NAD\(^+\) for KGDHC.

From the above equations it is evident that MAS directionality is governed by cytosolic [NAD\(^+\)], [NADH], mitochondrial [NAD\(^+\)], [NADH], \(\Delta\phi\), cytosolic [glutamate] and [aspartate] and mitochondrial [glutamate] and [aspartate]. It would be too complicated to graphically represent MAS as a function of all these parameters, but it can be deduced that MAS may tend to operate in reverse (removing reducing power of NADH from the matrix) when i) mitochondria are de-energized (low \(\Delta\Psi_{m}\)) ii) matrix NAD\(^+\)/NADH is low, and iii) cytosolic NAD\(^+\)/NADH is high. These considerations lend support to the suggestion that MAS may indeed operate in reverse during de-energized (i.e. anoxic) conditions, as suggested in (Bremer and Davis, 1975) and (Chouchani et al., 2014). Finally, it may be of value to acknowledge that in the cytosol NAD\(^+\) may not only originate from MDH1 or lactate dehydrogenase, but also from the process of desaturating fatty acids (Kim et al., 2019), alcohol dehydrogenases and aldehyde dehydrogenases (Cederbaum, 2012), increasing the likelihood for MAS reversibility.

9. Acute provision of NAD\(^+\) through NAD\(^+\) import across the inner mitochondrial membrane

The concept of MAS has been put forward exactly because NAD\(^+\) was considered impermeable to the inner mitochondrial membrane (Chappell, 1968), (Stein and Imai, 2012), whereas cytosolic and nuclear NAD\(^+\) pools are exchangeable via diffusion through connexin 43 and the nuclear pore, respectively (Bruzzone et al., 2001), (Verdin, 2015). However, in 1997 Rustin and colleagues reported that NAD\(^+\) is able to cross the inner mitochondrial membrane (Rustin et al., 1997). This has been more recently confirmed by the group of Baur using isotope labelling experiments (Davila et al., 2018), even though to date, no mechanism for direct NAD\(^+\) transport in mammalian mitochondria has been identified. Although the results are unequivocal, it begs the question why are there shuttles, if NAD\(^+\) can be directly imported; perhaps the rate of reducing equivalents appearing in the matrix of one mechanism over another is very different. Furthermore, this mechanism seems to be unidirectional, because mitochondria expressing a bacterial enzyme mediating strong NADH oxidation (mitoLbNOX, see under “artificial means of manipulating matrix NAD\(^+\)/NADH") drained reducing equivalents from the whole cell, while LbNOX expressed in the cytosol only oxidized the cytosol (Titov et al., 2016). By the same token, since mitoLbNOX, but not LbNOX, increased the mitochondrial NAD\(^+\)/
NADH ratio in HeLa cells, this means that import of NAD\(^+\) to mitochondria may not be quantitatively important, at least in this setting. It would have been of interest to perform experiments in LbNOX and mitoLbNOX-expressing cells when mitochondria are de-energized, and examine the extent of contribution of MAS reversibility in contributing the transfer of reducing power of pyridine nucleotides from the cytosol.

10. Artificial means of manipulating matrix NAD\(^+\)/NADH

In 2016, the group of Mootha published the results of expressing a water-forming NADH oxidase from Lactobacillus brevis (LbNOX) as a genetic tool for inducing a compartment-specific increase of the NAD\(^+\)/NADH ratio in human cells, which they also targeted to mitochondria (mitoLbNOX) (Titov et al., 2016). With this, they clearly demonstrated the compartment-specific manipulation of NAD\(^+\)/NADH ratio and its impact on redox-dependent events. By using this tool, it would be easy to address the role of [NAD\(^+\)] in metabolic settings directly, as opposed to manipulating electron transport chain which indirectly affects matrix NAD\(^+\)/NADH. Even more recently, the same group reported the engineering of a fusion of bacterial lactate oxidase (LOX) and catalase (CAT) named LOXCAT (Patgiri et al., 2020). This fusion enzyme exhibits the capacity of irreversibly converting lactate and oxygen to pyruvate and water and as an extension to that impact on intracellular NAD\(^+\)/NADH ratio. By injecting purified LOXCAT in living mice, beneficial changes in intracellular NAD\(^+\)/NADH ratio were observed in response to a metformin-induced rise in blood lactate/pyruvate ratio.

Also, the use of β-hydroxybutyrate vs acetoacetate in regulating matrix NAD\(^+\)/NADH ratio is a well-known and widely employed tool, through the reaction catalyzed by β-hydroxybutyrate dehydrogenase, see Fig. 2. Of course, this is only applicable for mitochondria expressing this enzyme, and in most circumstances the amount of β-hydroxybutyrate and/or acetoacetate used needs to be titrated (Chinopoulos et al., 2010), (Kiss et al., 2013), (Kiss et al., 2014), (Ravasz et al., 2018). The possibility of NAD\(^+\) originating from reverse activity of glutamate dehydrogenase, to the best of my knowledge, has never been reported; however, the substrate combination of α-ketoglutarate + malate was eliciting stronger mSLP than either glutamate + malate or α-ketoglutarate + glutamate + malate in isolated mitochondria, implying that NADH production by glutamate dehydrogenase from glutamate to α-ketoglutarate diminishes the availability of NAD\(^+\) for KGDCH (Chinopoulos et al., 2010), (Kiss et al., 2013), (Kiss et al., 2014), (Ravasz et al., 2018).

11. Conclusions

Hereby several acute sources of NADH oxidation are discussed; it must be born in mind that each of them may attain a condition-dependent role, and that some may be trivial. Furthermore, the present review addresses only the acute sources of NAD\(^+\) in the mitochondrial matrix; having said that, contribution of NAD\(^+\) from production pathways as reviewed by (Kulkarni and Brookes, 2019) and (Katsyuba et al., 2020) must not be ignored, but they can be considered as subacute and/or chronic means for yielding NAD\(^+\) simply because they are governed by slower kinetics.

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