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Reduction of 2-methoxy-1,4-naphtoquinone by mitochondrially-localized Nqo1 yielding NAD^+ supports substrate-level phosphorylation during respiratory inhibition☆

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ABSTRACT

Provision of NAD^+ for oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA by the ketoglutarate dehydrogenase complex (KGDHC) is critical for maintained operation of succinyl-CoA ligase yielding high-energy phosphates, a process known as mitochondrial substrate-level phosphorylation (mSLP). We have shown previously that when NADH oxidation by complex I is inhibited by rotenone or anoxia, mitochondrial diaphorases yield NAD^+, provided that suitable quinones are present (Kiss G et al., FASEB J 2014, 28:1682). This allows for KGDHC reaction to proceed and as an extension of this, mSLP. NAD(P)H quinone oxidoreductase 1 (NQO1) is an enzyme exhibiting diaphorase activity. Here, by using $Nqo1^{-/-}$ and WT littermate mice we show that in rotenone-treated, isolated liver mitochondria 2-methoxy-1,4-naphtoquinone (MNQ) is preferentially reduced by matrix Nqo1 yielding NAD^+ to KGDHC, supporting mSLP. This process was sensitive to inhibition by specific diaphorase inhibitors. Reduction of idebenone and its analogues MRQ-20 and MRQ-56, menadione, mitoquinone and duroquinone were unaffected by genetic disruption of the Nqo1 gene. The results allow for the conclusions that i) MNQ is a Nqo1-preferred substrate, and ii) in the presence of suitable quinones, mitochondrially-localized diaphorases other than Nqo1 support NADH oxidation when complex I is inhibited. Our work confirms that complex I bypass can occur by quinones reduced by intramitochondrial diaphorases oxidizing NADH, ultimately supporting mSLP. Finally, it may help to elucidate structure-activity relationships of redox-active quinones with diaphorase enzymes.

1. Introduction

Diaphorases are flavoenzymes catalyzing the oxidation of reduced pyridine nucleotides by endogenous or artificial electron acceptors. Since the purification of such an enzyme by Bruno Ferenc Straub in 1939 [1], other proteins were also shown to exhibit diaphorase activity, such as the DLD subunit of KGDHC [2–5]. “DT-diaphorase”, named after its ability to react with both DPNH (NADH) and TPNH (NADPH), discovered by Lars Ernster and colleagues [6] [7,8], is a diaphorase attributed to NAD(P)H quinone oxidoreductase 1 (NQO1) activity [9,10]. Although NQO1 is mostly considered a cytosolic enzyme, it –as well as DT-diaphorase activity with signatures similar to those of NQO1- has been shown to localize also in the mitochondrial matrix [7,11–20] (except in [21]). All reports showed that mitochondrial diaphorase activity accounted for < 15% of the total. However, despite the low activity for NAD(P)H oxidation, its localization to the mitochondrial matrix affords to the enzyme a special role in adverse circumstances: it is a textbook definition that NADH generated in the citric acid cycle by dehydrogenases is oxidized by complex I of the electron transport chain (ETC), leading to a sequence of events resulting in ATP production by the F_0–F_1 ATP synthase. However, during respiratory arrest or complex I inhibition by pharmacological or genetic means, NADH cannot be oxidized, and as we have shown recently, in isolated mitochondria from mouse liver up to 81% of NAD^+ pool can be regenerated by intramitochondrial diaphorases [10]. NAD^+ originating from diaphorases is then utilized by KGDHC forming succinyl-CoA, which is in turn converted to succinate by succinate-CoA ligase yielding ATP or GTP depending on subunit composition of the ligase [22–24]. In the absence of oxidative phosphorylation, ATP generated by succinate-CoA ligase prevents mitochondria from becoming cytosolic ATP consumers by maintaining the adenine nucleotide translocase in forward mode, i.e., expelling ATP to the cytosol in exchange for importing ADP

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to the matrix, thus avoiding a cellular bioenergetic catastrophe [25], [26,27], [24]. In freshly isolated, rotenone-treated (in the presence of glutamate and malate) mitochondria, provision of exogenous quinones for NADH oxidation by diaphorases is not critical due to the presence of sufficient amounts of endogenous quinones [10]; however, when the NADH/NAD^+ ratio is artificially elevated (i.e. by β-hydroxybutyrate), menadione or duroquinone greatly assist in the maintenance of mSLP implying provision of NAD^+ to KGDHC, and this process is sensitive to dicoumarol, chrysin, dihydroxyflavone and phenindione, all being specific diaphorase inhibitors [10].

Mindful that NQO1 localizes to mitochondria (even though accounting for < 15% of total cellular diaphorase activity), we were interested in the extent of contribution of this protein yielding NAD^+ in the matrix when complex I is inhibited. To characterize further the ability of mitochondrial Nqo1 oxidizing reducing equivalents, we tested the effect of a host of redox-active quinones and report that 2-methoxy-1,4-naphtoquinone (MNQ) is preferentially reduced by this flavoenzyme.

2. Materials and methods

2.1. Animals

Mice were of mixed 129Sv and C57Bl/6 background. Nqo1$^{-/-}$ mice were a kind gift of Dr. Frank J. Gonzalez. The animals used in our study were of either sex and between 2 and 6 months of age. Mice were housed in a room maintained at 20–22 °C on a 12-h light–dark cycle with food and water available ad libitum. All experiments were approved by the Animal Care and Use Committee of the Semmelweis University (Egyetemi Állatkísérleti Bizottság).

2.2. Isolation of mitochondria

Liver mitochondria were isolated as described in [28]. Protein concentration was determined using the bicinchoninic acid assay, and calibrated using bovine serum standards [29] using a Tecan Infinite® 200 PRO series plate reader (Tecan Deutschland GmbH, Crailsheim, Germany). Yields (in mg per two livers) were 16.32 ± 0.63 and 16.29 ± 1.59 (S.E.M.) for WT and NQO1$^{-/-}$ mice, respectively.

2.3. Determination of membrane potential in isolated mitochondria

ΔΨm of isolated mitochondria (0.5 or 1 mg for mouse liver per two ml of medium) was estimated fluorimetrically with safranine O [30] and calibrated to millivolts as described in [25], acknowledging the considerations elaborated in [31,32] regarding inhibition of respiration as well as unspecific binding of safranine. Substrates were either glutamate and malate, or glutamate and malate and β-hydroxybutyrate, or α-ketoglutarate and malate, or only α-ketoglutarate, all used at 5 mM concentration except β-hydroxybutyrate which was 2 mM.

Fluorescence was recorded in a Hitachi F-7000 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK) at a 5-Hz acquisition rate, using 495- and 585-nm excitation and emission wavelengths, respectively, or at a 1-Hz rate using the O2k-Fluorescence LED2-Module of the Oxygraph-2 k (Oroboros Instruments, Innsbruck, Austria) equipped with an LED exhibiting a wavelength maximum of 465 ± 25 nm (current for light intensity adjusted to 2 mA, i.e., level “4”) and an < 505 nm shortpass excitation filter (dye-based, filter set “Safranin”). Emitted light was detected by a photodiode (range of sensitivity: 350–700 nm), through an > 560 nm longpass emission filter (dye-based). Experiments were performed at 37 °C.

2.4. Mitochondrial respiration

Oxygen consumption was performed polarographically using an Oxygraph-2 k. 1 mg of liver mitochondria was suspended in 2 ml incubation medium, the composition of which was identical to that for ΔΨm determination, as described in [25]. Substrates were either glutamate and malate, or glutamate and malate and β-hydroxybutyrate, or α-ketoglutarate and malate, or only α-ketoglutarate, all used at 5 mM concentration except β-hydroxybutyrate which was 2 mM. Experiments were performed at 37 °C. Oxygen concentration and oxygen flux (pmol·s − 1 mg − 1; negative time derivative of oxygen concentration, divided by mitochondrial mass per volume and corrected for instrumental background oxygen flux arising from oxygen consumption of the oxygen sensor and back-diffusion into the chamber) were recorded using DatLab software (Oroboros Instruments).

2.5. Determination of NADH autofluorescence in permeabilized liver mitochondria

NADH autofluorescence was measured using 340 and 435 nm excitation and emission wavelengths, respectively. Measurements were performed in a Hitachi F-7000 fluorescence spectrophotometer at a 5 Hz acquisition rate. 0.5 mg of mouse liver mitochondria were suspended in 2 ml incubation medium, the composition of which was the following: 110 mM K-gluconate, 10 mM HEPES (free acid), 10 mM KH_2PO_4, 10 mM mannitol, 10 mM NaCl, 8 mM KCl, 1 mM $MgCl_2$, 0.01 mM EGTA, 0.5 mg/ml BSA (essentially fatty acid-free), with the pH adjusted to 7.25 with KOH. Substrates were either glutamate and malate, or glutamate and malate and β-hydroxybutyrate, or α-ketoglutarate and malate, or only α-ketoglutarate, all used at 5 mM concentration except β-hydroxybutyrate which was 2 mM. Experiments were performed at 37 °C.

2.6. Determination of diaphorase activity

NADH and NADPH, dicoumarol-sensitive diaphorase activity was measured by two different methods, one relying on 2,6-dichlorophenolindophenol (DCPIP) reduction [33] with the modifications detailed in [34] and the other on cytochrome *c* reduction [35]. For the latter method, the electron acceptor quinone was either MNQ, or menadione (MND) or duroquinone (DQ), and only NADH was used. Reduction of DCPIP was followed at 600 nm ($e = 21\ mM^{-1} * cm^{-1}$) and that of cytochrome *c* at 550 nm ($e = 18.5\ mM^{-1} * cm^{-1}$). Activities were determined by either method from the cytosolic and mitochondrial fractions from WT and Nqo1$^{-/-}$ mouse livers. In all cases, NADH and NADPH were used at 0.2 mM final concentration. Cytosolic fractions were obtained as detailed in [36]. Substrate concentrations are indicated in the legends. Measurements on mitochondrial fractions were performed in the presence of 2 μM rotenone. All assays were done at 30 °C.

2.7. Cell culturing

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotic solution (containing penicillin and streptomycin) at 37 °C in 5% CO_2. 300–350,000 cells were plated in 75 cm^2 culture flasks.

2.8. Mitochondrial membrane potential determination of in situ mitochondria of permeabilized HepG2 cells

Mitochondrial membrane potential (ΔΨm) was estimated using fluorescence quenching of the cationic dye safranin O due to its accumulation inside energized mitochondria [30]. Cells were harvested by scraping, permeabilized as detailed previously [37] and suspended in a medium identical to that as for ΔΨm measurements in isolated mitochondria. Substrates were 5 mM glutamate, 5 mM α-ketoglutarate and 5 mM malate. Fluorescence was recorded in a Tecan Infinite® 200 PRO series plate reader using 495 and 585 nm excitation and emission wavelengths, respectively. Experiments were performed at 37 °C.