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2-Oxoglutarate dehydrogenase is a more significant source of $O_2^{\cdot -}/H_2O_2$ than pyruvate dehydrogenase in cardiac and liver tissue

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ABSTRACT

Pyruvate dehydrogenase (Pdh) and 2-oxoglutarate dehydrogenase (Ogdh) are vital for Krebs cycle metabolism and sources of reactive oxygen species (ROS). $O_2^{\cdot -}/H_2O_2$ formation by Pdh and Ogdh from porcine heart were compared when operating under forward or reverse electron transfer conditions. Comparisons were also conducted with liver and cardiac mitochondria. During reverse electron transfer (RET) from NADH, purified Ogdh generated ~3–3.5 × more $O_2^{\cdot -}/H_2O_2$ in comparison to Pdh when metabolizing 0.5–10 μM NADH. Under forward electron transfer (FET) conditions Ogdh generated ~2–4 × more $O_2^{\cdot -}/H_2O_2$ than Pdh. In both liver and cardiac mitochondria, Ogdh displayed significantly higher rates of ROS formation when compared to Pdh. Ogdh was also a significant source of ROS in liver mitochondria metabolizing 50 μM and 500 μM pyruvate or succinate. Finally, we also observed that DTT directly stimulated $O_2^{\cdot -}/H_2O_2$ formation by purified Pdh and Ogdh and in cardiac or liver mitochondria in the absence of substrates and cofactors. Taken together, Ogdh is a more potent source of ROS than Pdh in liver and cardiac tissue. Ogdh is also an important ROS generator regardless of whether pyruvate or succinate serve as the sole source of carbon. Our observations provide insight into the ROS generating capacity of either complex in cardiac and liver tissue. The evidence presented herein also indicates DTT, a reductant that is routinely added to biological samples, should be avoided when assessing mitochondrial $O_2^{\cdot -}/H_2O_2$ production.

1. Introduction

Mitochondria are quantifiably one of the most important sources of ROS in mammalian cells [1]. The proximal ROS generated by mitochondria is superoxide ($O_2^{\cdot -}$) which is the result of univalent reduction of molecular oxygen (O_2). Following its production $O_2^{\cdot -}$ is dismutated rapidly by superoxide dismutase (SOD) generating hydrogen peroxide (H_2O_2). Flavoproteins are typically responsible for the premature reduction of O_2 which is related to the unique radical chemistry of flavin-based prosthetic groups [2,3]. Exceptions include ubiquinone (UQ) binding site in Complex I and III (specifically the Q_o site) which transfer one electron from the quinone pool to O_2 [4,5]. Both $O_2^{\cdot -}$ and H_2O_2 are considered mitochondrial signaling molecules. However, H_2O_2, which is referred to as a mitokine, is vital for coordinating mitochondrial function with changes in cell physiology [6,7]. Complex I and III of the respiratory chain are usually considered the main sites for mitochondrial ROS formation. However, it is now understood that mammalian mitochondria can also generate ROS from 10 other sites [8]. The different sites for ROS production can be subcategorized based on which cofactor, NADH or ubiquinol (UQH_2), is required to form $O_2^{\cdot -}$. The NAD^+/NADH isopotential group is composed of five enzymes; Complex I, branched chain keto acid dehydrogenase (Bckdh), 2-oxoadipate dehydrogenase (Dhtkd1), Pdh and Ogdh [8,9]. In depth analyses of the relative maximal contributions of each enzyme complex to overall $O_2^{\cdot -}/H_2O_2$ production in skeletal muscle mitochondria revealed that Pdh and Ogdh are major contributors to a mitochondrion's overall ROS yield [3,8]. Pdh and Ogdh were found to produce 4 and 8 times more ROS than Complex I [3]. Similar observations have been made with neuronal mitochondria where Ogdh produces more ROS than Pdh [10]. Previous studies have credited Complex I with higher than normal ROS production, especially when NADH was abundant. This assumption should be revised considering Pdh and Ogdh can also produce substantial amounts of $O_2^{\cdot -}/H_2O_2$ during forward or reverse electron flow [3,11–13].

Pdh and Ogdh occupy pivotal positions in energy metabolism connecting the degradation of carbohydrates and amino acids to mitochondrial ATP production. Pdh and Ogdh are composed of 3 different subunits which are required to couple the decarboxylation of pyruvate or 2-oxoglutarate to the reduction of NAD^+; pyruvate or 2-oxoglutarate decarboxylase (E_1), dihydrolipoamide

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transacetylase or dihydrolipoamide transsuccinylase (E_2), and dihydrolipoamide dehydrogenase (E_3) [14]. Pdh and Ogdh rely on several cofactors including thiamine pyrophosphate (TPP), CoASH, lipoamide, and flavin adenine dinucleotide (FAD), strategically located in the three subunits, to successfully transfer electrons to NAD^+. Oxidation of pyruvate or 2-oxoglutarate yields acetyl-CoA or succinyl-CoA, which are committed to further oxidation in the Krebs cycle, and NADH which is used to stimulate ATP biosynthesis. On top of its central function in metabolism, both enzymes are also important mitochondrial redox sensors. The lipoamide residue in the E_2 subunit of both enzymes harbors vicinal thiol residues that are amenable to oxidation by H_2O_2. It has been shown that lipoamide in Ogdh can be oxidized by low μM concentrations of H_2O_2 which deactivates the enzyme complex [15]. The enzyme is then reactivated through its reversible S-glutathionylation which also protects the lipoamide from irreversible oxidation or modification with electrophiles [15]. Similarly, Pdh has also been reported to be deactivated by ROS [16]. In addition, Pdh and Ogdh have recently been found to be regulated by changes in mitochondrial redox buffering networks [11,17]. Manipulation of mitochondrial glutathione (GSH) and glutathione disulfide (GSSG) levels alters $O_2^{\cdot -}/H_2O_2$ production by Pdh and Ogdh. $O_2^{\cdot -}/H_2O_2$ production by Pdh is drastically augmented if glutathione or NADPH pools are depleted [13]. Ogdh on the other hand senses changes in both GSH and GSSG with the former amplifying $O_2^{\cdot -}/H_2O_2$ formation and the latter having the opposing effect [11]. Overall, Pdh and Ogdh play intricate roles in redox sensing which is integrated heavily in the modulation of $O_2^{\cdot -}/H_2O_2$ formation and carbon metabolism.

Pdh and Ogdh are significant sources of $O_2^{\cdot -}/H_2O_2$. Here, we have performed a detailed biochemical study of the $O_2^{\cdot -}/H_2O_2$ producing capabilities of Pdh and Ogdh purified from porcine heart and in liver and cardiac mitochondria from C57Bl6J mice. Overall we found that Ogdh produces ~3–3.5× more $O_2^{\cdot -}/H_2O_2$ during reverse electron flow from NADH when compared to Pdh. During forward electron transfer, Ogdh was also the more significant source of ROS in cardiac and liver tissue. Serendipitously, we also observed that DTT, a reductant used widely in the study of mitochondrial bioenergetics, ROS production, and redox signaling, directly stimulated $O_2^{\cdot -}/H_2O_2$ by Pdh and Ogdh. The significance of these findings in relation to Pdh and Ogdh mediated sensing of mitochondrial redox buffering networks and control over ROS production are discussed herein.

2. Materials and methods

2.1. Reagents

Pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (α-ketoglutarate dehydrogenase) were of porcine heart origin and purchased from Sigma. H_2O_2 (30% solution), NAD^+, NADH, CoASH, TPP, mannitol, Hepes, sucrose, EGTA, fatty acid free bovine serum albumin, Bradford reagent, subtilisin A, Triton X-100, SOD, and horse radish peroxidase (HRP) were also purchased from Sigma. DTT was purchased from Bio-Rad and Amplex Ultra Red (AUR) reagent was acquired from Invitrogen.

2.2. Simultaneous Amplex Ultra Red and enzyme activity assays

$O_2^{\cdot -}/H_2O_2$ production and NADH production or consumption were monitored simultaneously [11,13]. For detection of H_2O_2 AUR was utilized in tandem with HRP. SOD was added to ensure complete conversion of $O_2^{\cdot -}$ to H_2O_2. To differentiate between whether or not $O_2^{\cdot -}$ or H_2O_2 was being produced directly by flavin centers in either enzyme, assays were conducted with AUR and HRP in the absence or presence of SOD. Prior to initiating reactions all substrates were allowed to equilibrate at room temperature for at least 1 min. Purified Pdh and Ogdh were diluted to 0.1 U/mL in MESH buffer (220 mM mannitol, 1 mM EGTA, 70 mM sucrose, and 20 mM Hepes, pH 7.4) and equilibrated for 5 min at room temperature. For forward electron transfer (FET) reactions, CoASH (0.1 mM), TPP (0.3 mM), NAD^+ (1 mM), AUR (10 μM), SOD (25 U/mL), and HRP (3 U/mL) were added to each well and assayed for enzyme activity and ROS production. The final volume for each reaction was 200 μL. Reactions were initiated by the addition of either pyruvate or 2-oxoglutarate to a final concentration of 0.01–20 mM. For RET reactions, pyruvate and 2-oxoglutarate, CoASH, TPP, and NAD^+ were excluded. Reactions were initiated by adding NADH to a final concentration of 0.01–200 μM.

DTT experiments were conducted as follows. Initial effects of DTT on Pdh and Ogdh were examined by pre-incubating enzymes in 1 mM DTT for 5 min followed by conducting assays for $O_2^{\cdot -}/H_2O_2$ and NADH production. To ascertain if DTT or GSH could support $O_2^{\cdot -}/H_2O_2$ formation by Pdh and Ogdh directly, either molecule was treated as a substrate. Reaction mixtures containing purified enzyme were treated as described above except CoASH, TPP, NAD^+ (unless specified otherwise) and pyruvate or 2-oxoglutarate were excluded from reaction mixtures. Reactions were then initiated by the addition of DTT (0.1–10 mM) or GSH (0.1–10 mM). For all reactions the final volume was 200 μL and the production of $O_2^{\cdot -}/H_2O_2$ and consumption of NADH were monitored simultaneously over 5 min at 24 °C at 30 s intervals. Reaction kinetics were recorded using a Synergy MX2 monochromatic microplate reader (BioTek). NADH and resorufin fluorescence was tracked at 376 nm:420 nm and 565 nm:600 nm respectively. $O_2^{\cdot -}/H_2O_2$ and NADH levels were quantified using standard curves and Gen5 software. Reaction conditions were adjusted to ensure that up to 8 wells can be monitored simultaneously in a 96 well black plate. All results were corrected for any background fluorescence.

2.3. Preparation of mitochondria

All animal experiments were approved by Memorial University's Animal Care and Use committee. All steps were performed on ice or at 4 °C unless stated otherwise. Mitochondria were enriched as described previously [18,19]. Livers and hearts were extracted from male C57BL/6N mice purchased from Charles River Laboratories. Mice (9 weeks old) were euthanized by cerebral dislocation under isoflurane anesthesia and livers and hearts were extracted and placed in MESH containing 0.5% (w/v) defatted BSA (MESH-B). For cardiac tissue, hearts were first cut into smaller pieces and washed with MESH-B to remove excess blood. The pieces were then minced and homogenized using the Potter-Elvjham method in 20 mL MESH-B containing 2 U of Subtilisin A. Subtilisin A is a protease that was added to ensure the release of intermyofibrillar mitochondria. Homogenates were then centrifuged at 10,000 × *g* for 9 min to remove Subtilisin A. The resulting pellet was resuspended in 20 mL MESH-B and centrifuged at 800 × *g* for 9 min to remove nuclei and intact tissue. The supernatant was placed in a second centrifuge tube and centrifuged at 10,000 × *g* for 9 min to yield a mitochondrial pellet. The pellet was resuspended in 100 μL MESH and stored on ice.

Livers were cut into smaller pieces and then washed in MESH-B to remove excess blood and fat. Pieces were then minced and homogenized in MESH-B. The homogenate was then centrifuged at 800 × *g* for 9 min. The supernatant was collected and centrifuged at 10,000 × *g* for 9 min. The resulting mitochondrial pellet was then resuspended in 1 mL of MESH and stored on ice. Mitochondrial protein concentration was determined using Bradford assays with BSA as the standard.