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Original article

Protein S-glutathionylation alters superoxide/hydrogen peroxide emission from pyruvate dehydrogenase complex

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ABSTRACT

Pyruvate dehydrogenase (Pdh) is a vital source of reactive oxygen species (ROS) in several different tissues. Pdh has also been suggested to serve as a mitochondrial redox sensor. Here, we report that $O_2^{\bullet-}$/ H_2O_2 emission from pyruvate dehydrogenase (Pdh) is altered by S-glutathionylation. Glutathione disulfide (GSSG) amplified $O_2^{\bullet-}$/ H_2O_2 production by purified Pdh during reverse electron transfer (RET) from NADH. Thiol oxidoreductase glutaredoxin-2 (Grx2) reversed these effects confirming that Pdh is a target for S-glutathionylation. S-glutathionylation had the opposite effect during forward electron transfer (FET) from pyruvate to NAD^+ lowering $O_2^{\bullet-}$/ H_2O_2 production. Immunoblotting for protein glutathione mixed disulfides (PSSG) following diamide treatment confirmed that purified Pdh can be S-glutathionylated. Similar observations were made with mouse liver mitochondria. S-glutathionylation catalysts diamide and disulfiram significantly reduced pyruvate or 2-oxoglutarate driven $O_2^{\bullet-}$/ H_2O_2 production in liver mitochondria, results that were confirmed using various Pdh, 2-oxoglutarate dehydrogenase (Ogdh), and respiratory chain inhibitors. Immunoprecipitation of Pdh and Ogdh confirmed that either protein can be S-glutathionylated by diamide and disulfiram. Collectively, our results demonstrate that the S -glutathionylation of Pdh alters the amount of ROS formed by the enzyme complex. We also confirmed that Ogdh is controlled in a similar manner. Taken together, our results indicate that the redox sensing and ROS forming properties of Pdh and Ogdh are linked to S-glutathionylation.

1. Introduction

Protein S-glutathionylation involves the conjugation of glutathione to a cysteine residue which is vital for controlling protein function and protecting protein thiols from irreversible oxidation [1,2]. These reactions are highly responsive to changes in cellular H_2O_2 and NADPH which alter the reductive state of glutathione pools and the S-glutathionylated proteome [3]. Protein S-glutathionylation reactions are highly specific, rapid, and reversible owing to the fact that the forward and reverse reactions are mediated by thiol oxidoreductase glutaredoxin (Grx) and glutathione S-transferases and that proteins have S-glutathionylation motifs [4,5]. In the cytosol, Grx1 catalyzes the S-glutathionylation and deglutathionylation of a number of protein targets involved in glycolysis, calcium homeostasis, energy sensing, apoptosis and transcriptional regulation [6,7]. Mitochondria also harbor a number of S-glutathionylation targets which includes Krebs cycle enzymes, respiratory complexes I, II and V, solute anion carrier proteins, factors involved in mitochondrial fission and fusion, apoptosis, and protein import machinery [3]. Grx2 is responsible for catalyzing reversible S-glutathionylation reactions in mitochondria which has been found to play an important part in controlling mitochondrial bioenergetics and reactive oxygen species (ROS) production [8,9]. The importance of protein S-glutathionylation in mitochondria is underscored by the pathological consequences associated with deregulating these reactions. Loss of Grx2 is associated with development of cardiac hypertrophy and fibrosis, results in the formation of cataracts, and hinders embryonic development which is associated with impaired oxidative phosphorylation and higher than normal ROS formation [8,10,11]. By contrast, preservation or restoration of protein S-glutathionylation reactions in mitochondria improves ATP production and prevents apoptosis [8,12]. Only a handful of Grx2 targets have been identified to date which includes Complex I of the respiratory chain and potentially uncoupling protein-3 and Ogdh [13–15]. Although it has been found that protein S-glutathionylation can affect overall mitochondrial ROS production in different tissues, the impact of these reactions on the individual proteins involved in mitochondrial $O_2^{\bullet-}$/ H_2O_2 production remains poorly resolved. Indeed, mitochondria contain 11 potential ROS generating sites and it has been found that S-

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glutathionylation is required to alter $O_2^{\bullet-}$/ H_2O_2 emission from Complex I and Ogdh [9,15,16].

Pdh and Ogdh represent major entry points for carbon into the Krebs cycle. Pdh is integral for committing the glycolytic degradation product of carbohydrate metabolism, pyruvate, to oxidation in the Krebs cycle. Ogdh on the other hand as that connects amino acid metabolism to Krebs cycle flux. Both enzymes are composed of three different subunits; pyruvate or 2-oxoglutarate decarboxylase (E_1), dihydrolipoamide transacetylase or dihydrolipoamide transsuccinylase (E_2), and dihydrolipoamide dehydrogenase (E_3), all of which are required to form NADH from the oxidation of pyruvate or 2-oxoglutarate [17]. Successful production of NADH also requires several prosthetic groups and cofactors, namely, thiamine pyrophosphate (TPP), dihydrolipoamide, and flavin adenine dinucleotide (FAD) which are housed in the E_1, E_2, and E_3 subunits respectively [18]. Pdh and Ogdh are also sources of mitochondrial ROS. Pdh and Ogdh have been found to produce ~4 and ~8 times more ROS than Complex I and have been shown to be important $O_2^{\bullet-}/H_2O_2$ generators in liver, cardiac, skeletal muscle, and brain mitochondria [17,19,20]. In addition, Pdh and Ogdh purified from porcine heart or human Pdh or Ogdh expressed and purified from bacteria are able to produce $O_2^{\bullet-}/H_2O_2$ via reverse electron transfer (RET) from NADH [20,21]. Thus, Pdh and Ogdh are contributors to overall mitochondrial $O_2^{\bullet-}/H_2O_2$ emission in various tissues suggesting that either enzyme may play a critical role in mediating mitochondrial redox signals to the rest of the cell.

Ogdh is subjected to reversible S-glutathionylation which is required to protect the enzyme complex from irreversible oxidative deactivation [22]. Our group has recently extended on these seminal findings by showing that fluctuations in the glutathione redox buffering network modulates the amount of ROS generated by Ogdh through S-glutathionylation [15]. In addition Fisher-Wellman et al. found that changes in glutathione redox buffering networks also modulates ROS emission from Pdh [23]. However, whether or not Pdh can be S-glutathionylated and if this redox modification alters ROS emission from this enzyme has remained unexplored. Here, we present evidence indicating that Pdh can be S-glutathionylated which affects the ROS-generating potential of the enzyme complex. We found that Pdh is S-glutathionylated on its E_2 subunit which is also an S-glutathionylation target in Ogdh. S-glutathionylation of Pdh amplifies RET driven ROS production. However, modification of Pdh with glutathione has the opposite effect with respect to ROS production when pyruvate is being oxidized. The results herein also show that chemical induction of the S-glutathionylation of Pdh and Ogdh has the same effect in mouse liver mitochondria. Diamide and disulfiram treatment significantly lowered $O_2^{\bullet-}/H_2O_2$ production by either enzyme indicating that there is a common mechanism for the redox regulation of ROS emission from Pdh and Ogdh. The importance of these findings in the context of controlling mitochondrial ROS signaling are discussed herein.

2. Materials and methods

2.1. Chemicals

Pyruvate dehydrogenase was of porcine heart origin and purchased from Sigma. Grx2 (human origin), glutathione reductase (GR), hydroxyethyl disulfide (HEDS), GSH, GSSG, diamide, disulfiram, H_2O_2 (30% solution), NAD^+, NADH, NADPH, CoASH, TPP, mannitol, Hepes, sucrose, EGTA, fatty acid free bovine serum albumin, Bradford reagent, Triton X-100, SOD, myxothiazol, 3-methyl-2-oxopentanoic acid (KMV), CPI-613, and horse radish peroxidase (HRP) were purchased from Sigma. Amplex Ultra Red (AUR) reagent, Dynabeads, and biotinylated glutathione ethylester (BioGEE) were acquired from Invitrogen. Pdh immunoprecipitation kit was purchased from MitoSciences. Protein glutathione mixed disulfide (PSSG), Ogdh, and Pdh subunit cocktail anti-serum were purchased from Abcam. Anti-mouse and anti-rabbit horseradish peroxidase secondary antibodies and atpenin A5 were purchased from Santa Cruz.

2.2. Simultaneous measurement of $O_2^{\bullet-}/H_2O_2$ and NADH by purified Pdh

$O_2^{\bullet-}/H_2O_2$ production and NADH production or consumption were monitored simultaneously [15,23]. For detection of H_2O_2 AUR was utilized in tandem with HRP. SOD was added to ensure complete conversion of $O_2^{\bullet-}$ to H_2O_2. However, it should be emphasized that the primary ROS generated by Pdh and Ogdh is H_2O_2 [20]. All substrates were allowed to equilibrate at room temperature for at least 1 min. Purified Pdh was 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), equilibrated for 5 min at room temperature, and then incubated for 15 min at 25 °C in GSSG (0.01–10 mM), or diamide (0–2000 μM) + GSH (1 mM). It is important to emphasize that porcine Pdh purity was tested previously [20]. 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 to a final concentration of 10 mM. For RET reactions, pyruvate, CoASH, TPP, and NAD^+ were excluded. Reactions were initiated by adding NADH to a final concentration of 0.01–100 μM. For experiments where Grx2 was added, prior to initiating reactions with NADH Grx2 and GSH were added to a final concentration of 0.5 μM and 0.5 mM, respectively. The H_2O_2 dependent conversion of AUR to fluorescent resorufin was tracked at ex:em 565 nm: 600 nm and production or consumption of NADH was measured by its autofluorescence at em:ex 376 nm: 450 nm using Synergi MX2 monochromatic microplate reader (Biotek). All results were normalized to amount of Pdh enzyme used (units of enzyme) and expressed as either pmol H_2O_2 min^{-1} U^{-1} or nmol NADH min^{-1} U^{-1} as reported previously [23].

2.3. Grx2 activity assay

Hydroxyethyl disulfide (HEDS) assay was used to examine Grx2 activity [12]. HEDS was diluted to 1.8 mM in MESH with GSH (0.9 mM) and incubated at 25 °C for 30 min to generate a hydroxyethyl-glutathione mixed disulfide. Reaction mixtures were then supplemented with Grx2 (0.5 μM) and GR (0.4 U/mL) followed by the addition of GSH (1 mM) and NADPH (0.2 mM). Reactions were monitored over 5 min in 30 s intervals. NADPH consumption was monitored by autofluorescence (em:ex, 376 nm: 450 nm) with a Synergi MX2 monochromatic microplate reader (Biotek). To ascertain if Pdh can be deglutathionylated by Grx2, Pdh was incubated in GSSG (1 mM) for 15 min at 25 °C to induce its S-glutathionylation. Reactions were stopped with 10 mM N-ethylmaleimide (NEM) and then excess GSSG and NEM was removed using PD10 desalting columns (GE Life Sciences). The eluent was collected and GR and Grx2 were then added to the reaction mixture followed by NADPH. Grx2 activity was then tracked over 5 min.

2.4. Preparation of liver mitochondria

All animal experiments were approved by Memorial University's Animal Care and Use committee. All steps were performed on ice or 4 °C. Mitochondria were enriched from the livers of male C57BL/6N mice purchased from Charles River Laboratories. Mice (9–11 weeks old) were euthanized by cerebral dislocation under isoflurane anesthesia and livers were placed in MESH supplemented with 0.5% fatty acid free BSA (MESH-B). Livers were cut into small pieces, washed several times, and homogenized in 15 mL MESH-B using the Potter-Elvejham method. Homogenates were centrifuged at 800 × *g* for 9 min after which fat was removed from the top of the supernatant. The supernatant was collected and centrifuged at 10,000 × *g* for 9 min to pellet mitochondria. The supernatant was decanted, excess fat was wiped clean from the sides of