Free Radical Biology and Medicine 81 (2015) 119–127

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Reduction of mitochondrial protein mitoNEET [2Fe–2S] clusters by human glutathione reductase

Aaron P. Landry, Zishuo Cheng, Huangen Ding*

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

ARTICLE INFO

Article history:
Received 7 November 2014
Received in revised form
9 January 2015
Accepted 21 January 2015
Available online 30 January 2015

Keywords:
MitoNEET
Type 2 diabetes
Iron–sulfur cluster
Thioredoxin reductase
Glutathione reductase
Free radicals

ABSTRACT

The human mitochondrial outer membrane protein mitoNEET is a newly discovered target of the type 2 diabetes drug pioglitazone. Structurally, mitoNEET is a homodimer with each monomer containing an N-terminal transmembrane α helix tethered to the mitochondrial outer membrane and a C-terminal cytosolic domain hosting a redox-active [2Fe–2S] cluster. Genetic studies have shown that mitoNEET has a central role in regulating energy metabolism in mitochondria. However, the specific function of mitoNEET remains largely elusive. Here we find that the mitoNEET [2Fe–2S] clusters can be efficiently reduced by *Escherichia coli* thioredoxin reductase and glutathione reductase in an NADPH-dependent reaction. Purified human glutathione reductase has the same activity as *E. coli* thioredoxin reductase and glutathione reductase to reduce the mitoNEET [2Fe–2S] clusters. However, rat thioredoxin reductase, a human thioredoxin reductase homolog that contains selenocysteine in the catalytic center, has very little or no activity to reduce the mitoNEET [2Fe–2S] clusters. *N*-ethylmaleimide, a potent thiol modifier, completely inhibits human glutathione reductase from reducing the mitoNEET [2Fe–2S] clusters, indicating that the redox-active disulfide in the catalytic center of human glutathione reductase may be directly involved in reducing the mitoNEET [2Fe–2S] clusters. Additional studies reveal that the reduced mitoNEET [2Fe–2S] clusters in mouse heart cell extracts can be reversibly oxidized by hydrogen peroxide without disruption of the clusters, suggesting that the mitoNEET [2Fe–2S] clusters may undergo redox transition to regulate energy metabolism in mitochondria in response to oxidative signals.

Thiazolidinediones (TZDs)[1] such as pioglitazone are prescription drugs for patients with type 2 diabetes [1]. Whereas the primary target of TZDs is the peroxisome proliferator-activated receptor γ (PPARγ), which regulates the expression of the genes for fatty acid metabolism and insulin signaling pathways [2], TZDs also have PPARγ-independent physiological effects on energy metabolism in mitochondria [3]. This observation led to the discovery of a novel mitochondrial outer membrane protein, mitoNEET, which specifically binds pioglitazone [4]. Deletion of mitoNEET in mice decreases the oxidative phosphorylation capacity in mitochondria [5]. On the other hand, increased expression of mitoNEET in adipocytes enhances lipid uptake and storage and inhibits mitochondrial iron transport into the matrix [6], suggesting that mitoNEET may regulate energy metabolism in mitochondria [4]. Recent studies further showed that mitoNEET has a central role in the development of neurodegenerative diseases [7], breast cancer proliferation [8], TNFα-induced necroptosis in hepatocytes [9], and browning of white adipose tissue [10], among other pathological conditions [11].

Human mitoNEET is a homodimer with each monomer containing an N-terminal transmembrane α helix (residues 14 to 32) tethered to the mitochondrial outer membrane [5] and a C-terminal cytosolic domain hosting a redox-active [2Fe–2S] cluster via the unusual ligand arrangement of three cysteines (Cys-72, Cys-74, and Cys-83) and one histidine (His-87) [12–15]. Whereas the specific function of mitoNEET is not fully understood, several studies have suggested that mitoNEET may transfer its [2Fe–2S] clusters to apo-ferredoxin *in vitro* [16,17] or to the iron-regulatory protein-1 in vivo [18]. Because mitochondria are the primary sites for iron–sulfur cluster biogenesis [19], it was a compelling suggestion that mitoNEET may act as a carrier, transporting iron–sulfur clusters assembled in mitochondria to target proteins in the cytoplasm [16–18]. Nevertheless, the observed cluster transfer occurs only when the mitoNEET [2Fe–2S] clusters are in oxidized state [16]. Because the redox midpoint potential of the mitoNEET [2Fe–2S] clusters (E_{m7}) is 0 mV [20,21] and the cytosolic redox potential is approximately −325 mV (pH 7.0) [22], the mitoNEET [2Fe–2S] clusters are mostly in the reduced state in cells under normal physiological conditions [23]. Thus, mitoNEET may transfer its iron–sulfur clusters to target proteins only when cells are under oxidative stress conditions [16].

Abbreviations: E_{m7}, redox midpoint potential at pH 7.0; EPR, electron paramagnetic resonance; GSSG, oxidized glutathione; GSR, glutathione reductase; mito, human mitoNEET; NEM, *N*-ethylmaleimide; TZD, thiazolidinedione

* Corresponding author.
E-mail address: hding@lsu.edu (H. Ding).

http://dx.doi.org/10.1016/j.freeradbiomed.2015.01.017
0891-5849/

Alternatively, mitoNEET may directly regulate energy metabolism in mitochondria via its redox-active [2Fe–2S] clusters [20,23]. It has already been reported that the redox property of the mitoNEET [2Fe–2S] clusters can be modulated by pH [21], interdomain interactions [24,25], the hydrogen bond network [26], the type 2 diabetes drug pioglitazone [20], and $NADP^+$/NADPH [17,27]. Recent proteomic studies further suggested that mitoNEET may form complexes with proteins that are crucial in aging, diabetes, and neurodegenerative diseases [28,29]. Thus, the mitoNEET [2Fe–2S] cluster may act as a sensor of multiple cellular signals to regulate mitochondrial functions via specific protein–protein interactions. In previous studies, we reported that the mitoNEET [2Fe–2S] clusters are fully reduced when expressed in *Escherichia coli* cells and that purified mitoNEET [2Fe–2S] clusters can be reduced by dithiothreitol or the *E. coli* thioredoxin/thioredoxin reductase system [23]. However, specific components that reduce or oxidize the mitoNEET [2Fe–2S] clusters in mammalian cells have not been identified. Here, we find that human mitoNEET [2Fe–2S] clusters can be efficiently reduced by *E. coli* thioredoxin reductase and glutathione reductase in an NADPH-dependent reaction. Purified human glutathione reductase [30] has the same activity as *E. coli* glutathione reductase to reduce the mitoNEET [2Fe–2S] clusters. However, rat thioredoxin reductase, a human thioredoxin reductase homolog that contains an unusual selenocysteine residue (U498) in the catalytic center [31–33], has very little or no activity to reduce the mitoNEET [2Fe–2S] clusters. *N*-ethylmaleimide, a potent modifier of redox-active thiols in human glutathione reductase [34], completely inhibits the enzyme from reducing the mitoNEET [2Fe–2S] clusters, indicating that the redox-active disulfide in the catalytic center of human glutathione reductase may be directly involved in reducing the mitoNEET [2Fe–2S] clusters. Additional studies show that the reduced mitoNEET [2Fe–2S] clusters in the mouse heart cell extracts can be reversibly oxidized by hydrogen peroxide. The results led us to propose that the mitoNEET [2Fe–2S] clusters may act as a novel redox sensor to modulate energy metabolism in mitochondria in response to oxidative signals.

Materials and methods

Protein preparation

A DNA fragment encoding human $mitoNEET_{33–108}$ (containing amino acid residues 33–108) was synthesized (Genscript Co.) and cloned into pET28b+. Recombinant mitoNEET was expressed in *E. coli* BL21/DE3 strain and purified as described previously [23,35]. *E. coli* thioredoxin reductase [36], glutathione reductase [37], succinic semialdehyde dehydrogenase [38], and 2,4-dienoyl-CoA reductase [39] were prepared using the *E. coli* strains from the ASKA library [40]. Recombinant human glutathione reductase was prepared using plasmid pUB302 (kindly provided by Professor Katja Becker, Justus Liebig University, Germany) in the *E. coli* strain SG5 (in which the gene *gor* encoding glutathione reductase was deleted) [41]. Briefly, *E. coli* strain SG5 cells hosting pUB302 were grown in LB medium at 37 °C for 5 h without inducers, harvested, and disrupted by passing through a french press once. The crude cell extracts were centrifuged at 12,000*g* for 30 min at 4 °C to remove cell debris. The supernatant was dialyzed against 20 mM Tris for 5 h at 4 °C. Then 1 mM oxidized glutathione (GSSG) was added to the dialyzed sample, which was loaded onto an ADP–Sepharose column and washed with 0.3 M NaCl. Human glutathione reductase was then eluted from the column with 0.8 M NaCl. The purity of the purified proteins was greater than 95% as judged by electrophoresis analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie blue. The protein concentrations of human mitoNEET, *E. coli* thioredoxin reductase, *E. coli* glutathione reductase, and human glutathione reductase were measured at 280 nm using extinction coefficients of 8.6, 17.7, 38.2, and 34.9 $mM^{-1}\,cm^{-1}$, respectively. Native rat liver thioredoxin reductase was purchased from Sigma. Recombinant rat thioredoxin reductase (containing native selenocysteine in the catalytic center) [42] was purchased from Cayman Chemical.

Cell extract preparation from E. coli cells and mouse heart

Wild-type *E. coli* cells (BL21/DE3) were grown in LB medium at 37 °C under aerobic conditions to OD at 600 nm of 1.0. Cells were harvested, washed with buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0), and passed through a french press once. After centrifugation at 8000*g* for 30 min at 4 °C to remove cell debris, the supernatant was loaded onto a Hi-Trap Desalting column (GE Lifesciences). For the mouse heart cell extracts, hearts from wild-type mice (C57BL/6 J, 3–4 weeks of age, obtained from the Pennington Biomedical Research Center, Louisiana State University) were cleaned in phosphate-buffered saline and minced with scissors. Heart tissues were ground with a homogenizer and further disrupted by drawing and ejecting samples using a syringe with a 26-gauge needle. Cell debris was removed by centrifugation at 8000*g* for 20 min at 4 °C, and the supernatant was used for the experiments. The protein concentration of the prepared cell extracts was determined using the Bradford assay [43].

Analyses of the redox state of the mitoNEET [2Fe–2S] clusters

Purified human mitoNEET dissolved in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) in a sealed vial was purged with pure argon gas for 15 min. The prepared cell extracts or enzymes were also purged with pure argon gas before being transferred to the sealed vials using a gas-tight Hamilton syringe. The reaction solutions were then incubated in a 37 °C water bath for the indicated times before the samples were analyzed by using the Beckman DU640 UV–visible spectrometer or EPR (electron paramagnetic resonance). For the NEM (*N*-ethylmaleimide) treatments, purified human glutathione reductase or the mouse heart cell extracts were preincubated with NEM (2 mM) and NADPH (0.2 mM) at room temperature for 3 hours to block the redox-active monothiols in the proteins.

EPR measurements

The X-band EPR spectra were recorded using a Bruker Model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous-flow cryostat. Routine EPR conditions were microwave frequency, 9.47 GHz; microwave power, 10.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 30 K; receiver gain, 2×10^5. The amount of the reduced mitoNEET [2Fe–2S] clusters was quantified as in [23].

Chemicals

Isopropyl β-D-1-thiogalactopyranoside, NADPH, kanamycin, ampicillin, and dithiothreitol were purchased from Research Products International Co. Reduced glutathione, oxidized glutathione, *N*-ethylmaleimide, hydrogen peroxide, and other chemicals were purchased from Sigma Chemical Co.

Results

Reduction of the mitoNEET [2Fe–2S] clusters by E. coli thioredoxin reductase and glutathione reductase

When recombinant human mitoNEET was expressed in *E. coli* cells, the mitoNEET [2Fe–2S] clusters were fully reduced [23], indicating