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

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Abstract

Human mitochondrial outer membrane protein mitoNEET is a newly discovered target of type II diabetes drug pioglitazone. Structurally, mitoNEET is a homodimer with each monomer containing an N-terminal transmembrane alpha helix tethered to 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, 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 to reduce 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.

Keywords

mitoNEET; type II diabetes; iron-sulfur cluster; thioredoxin reductase; glutathione reductase

INTRODUCTION

Thiazolidinediones (TZDs) such as pioglitazone are prescription drugs for patients with type II diabetes [1]. While the primary target of TZDs is the peroxisome proliferator-activated

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receptor γ (PPAR γ) which regulates the expression of the genes for fatty acid metabolism and insulin signaling pathways [2], TZDs also have the PPAR γ -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 development of neurodegenerative diseases [7], breast cancer proliferation [8], TNF α -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 mitochondrial outer membrane [5] and a C-terminal cytosolic domain hosting a redox active [2Fe-2S] cluster via the unusual ligand arrangement of three cysteine (Cys-72, Cys-74 and Cys-83) and one histidine (His-87) residues [12–15]. While the specific function of mitoNEET has not been 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 (IRP-1) *in vivo* [18]. Since mitochondria are the primary sites for iron-sulfur cluster biogenesis [19], it was compelling to suggest that mitoNEET may act as a carrier transporting iron-sulfur clusters assembled in mitochondria to target proteins in cytoplasm [16–18]. Nevertheless, the observed cluster transfer occurs only when the mitoNEET [2Fe-2S] clusters are in oxidized state [16]. Since the redox midpoint potential of the mitoNEET [2Fe-2S] clusters (E_{m7}) is 0 mV [20, 21] and the cytosolic redox potential is ~ -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].

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], inter-domain interactions [24, 25], hydrogen bond network [26], type II 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 to reduce 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

1. 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 an *E. coli* strain SG5 (in which gene *gor* encoding glutathione reductase was deleted) [41]. Briefly, *E. coli* strain SG5 cells hosting pUB302 were grown in LB media at 37°C for 5 hours without inducers, harvested, and disrupted by passing through French press once. The crude cell extracts were centrifuged at 12,000g for 30 min at 4°C to remove cell debris. Supernatant was dialyzed against 20 mM Tris for 5 hours at 4°C. The dialyzed sample was added with 1 mM oxidized glutathione (GSSG), and 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 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 an extinction coefficient of 8.6 mM⁻¹ cm⁻¹, 17.7 mM⁻¹ cm⁻¹, 38.2 mM⁻¹ cm⁻¹, 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.

2. Cell extracts preparation from *E. coli* cells and mouse heart

Wild type *E. coli* cells (BL21/DE3) were grown in LB media 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 French press once. After centrifugation at 8,000 × g for 30 min at 4°C to remove cell debris, supernatant was loaded onto Hi-Trap Desalting column (GE Lifesciences). For the mouse heart cell extracts, hearts

from wild-type mice (C57BL/6J, 3–4 weeks old, obtained from the Pennington Biomedical Research Center, Louisiana State University) were cleaned in PBS buffer 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 were removed by centrifugation at $8,000 \times g$ 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 Bradford assay [43].

3. 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 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 time 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 pre-incubated with NEM (2 mM) and NADPH (0.2 mM) at room temperature for 30 min to block the redox active monothiol in the proteins.

4. EPR measurements

The X-band EPR (Electron Paramagnetic Resonance) 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; receive gain, 2×10^5 . The amount of the reduced mitoNEET [2Fe-2S] clusters was quantified as in [23].

5. Chemicals

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

RESULTS

1. 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 that *E. coli* cells have a robust activity to reduce the mitoNEET [2Fe-2S] clusters. To search for the cellular components that are responsible for reducing the human mitoNEET [2Fe-2S] clusters, we prepared cell extracts from exponentially growing *E. coli* cells. Figure 1A shows that while NADPH (spectrum 2) or the *E. coli* cell extracts (spectrum 3) had no activity to reduce the human mitoNEET [2Fe-2S] clusters, addition of NADPH to the cell extracts quickly reduced the mitoNEET [2Fe-2S] clusters (spectrum 4) which had a typical rhombic EPR (Electron Paramagnetic

Resonance) signal at $g = 1.94$ [12, 23, 26]. The amplitude of the EPR signal at $g = 1.94$ of the mitoNEET [2Fe-2S] clusters in the *E. coli* cell extracts (spectrum 4) was essentially the same as that when the mitoNEET protein was reduced with sodium dithionite (spectrum 5), indicating that the mitoNEET [2Fe-2S] clusters are fully reduced in the *E. coli* cell extracts by NADPH.

Since the mitoNEET [2Fe-2S] clusters can be reduced by excess dithiothreitol *in vitro* [23], we reasoned that the mitoNEET [2Fe-2S] clusters may be reduced by reduced thioredoxin [23] or the enzymes that contain redox active disulfide *in vivo*. In *E. coli*, there are at least two reductases: thioredoxin reductase [44] and glutathione reductase [37], that have a redox active disulfide in the catalytic center. To test this idea, we prepared *E. coli* thioredoxin reductase and glutathione reductase as described in the Materials and Methods. Purified *E. coli* thioredoxin reductase and glutathione reductase had a similar UV-visible absorption spectrum (Figure 1B), indicative of the protein-bound FAD as reported previously [37, 44].

Figure 1C shows that purified *E. coli* thioredoxin reductase (spectrum 3) or glutathione reductase (spectrum 4) had no activity to reduce the mitoNEET [2Fe-2S] clusters. However, when mitoNEET was incubated with *E. coli* thioredoxin reductase and NADPH (spectrum 5) or *E. coli* glutathione reductase and NADPH (spectrum 6) at 37°C for 20 min under anaerobic conditions, the mitoNEET [2Fe-2S] clusters were fully reduced, suggesting that both *E. coli* thioredoxin reductase and glutathione reductase are able to reduce the mitoNEET [2Fe-2S] clusters in the presence of NADPH. In parallel, we also prepared two NADPH-dependent reductases: succinic semialdehyde dehydrogenase [38] and 2,4-dienoyl-CoA reductase [39] from *E. coli* cells. Both enzymes use NADPH as substrate but do not contain the redox active disulfide in the catalytic center. Under the same experimental conditions, *E. coli* succinic semialdehyde dehydrogenase and 2,4-dienoyl-CoA reductase had no activity to reduce the mitoNEET [2Fe-2S] clusters in the presence of NADPH (data not shown). Thus, *E. coli* thioredoxin reductase and glutathione reductase have the specific activity in reducing the human mitoNEET [2Fe-2S] clusters.

2. Reduction of the mitoNEET [2Fe-2S] clusters by human glutathione reductase

In humans, there are three thioredoxin reductases: a cytosolic form [45], a mitochondrial form [46], and a thioredoxin glutathione reductase in the microsomal fraction of testis tissue [47]. As mitoNEET localizes on mitochondrial outer membrane [5], it is most likely that cytosolic thioredoxin reductase would be responsible for reducing the mitoNEET [2Fe-2S] clusters. Like *E. coli* thioredoxin reductase, human cytosolic thioredoxin reductase contains FAD and an NADPH binding site [32, 33, 48]. However, unlike *E. coli* thioredoxin reductase, human thioredoxin reductase has an unusual selenocysteine residue (U498) in the catalytic center [31–33]. As native human cytosolic thioredoxin reductase was not readily available, we used rat thioredoxin reductase which has 91% identity and 96% similarity to human thioredoxin reductase and contains the selenocysteine residue in the catalytic center. Rat liver thioredoxin reductase (from Sigma) was fully active to reduce 5,5'-dithiobis-(2-nitrobenzoic acid). However, unlike *E. coli* thioredoxin reductase, rat liver thioredoxin reductase had very little or no activity to reduce the mitoNEET [2Fe-2S] clusters (data not shown). We also obtained recombinant rat thioredoxin reductase (from Cayman Chemical)

which also contains the selenocysteine residue and is fully active to reduce 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [42]. Again, recombinant rat thioredoxin reductase failed to reduce the mitoNEET [2Fe-2S] clusters in the presence of NADPH (Figure 2B, spectrum 2).

Human glutathione reductase is a major cellular reductase to reduce the oxidized glutathione in cytosol [30, 41]. Unlike human thioredoxin reductase, human glutathione reductase does not have selenocysteine in the catalytic center. We thus prepared recombinant human glutathione reductase from *E. coli* cells following the procedures described in [41]. Purified human glutathione reductase had a similar UV-visible absorption spectrum as *E. coli* glutathione reductase (Figure 2A), and similar activity to reduce the mitoNEET [2Fe-2S] clusters in the presence of NADPH (Figure 2B, spectra 3 and 4). Thus, in human cells, at least glutathione reductase is able to reduce the mitoNEET [2Fe-2S] clusters in an NADPH-dependent reaction.

Pioglitazone, a type II diabetes drug, has been shown to shift the redox midpoint potential (E_{m7}) of the mitoNEET [2Fe-2S] clusters by ~ -100 mV [20]. The negative shift of the E_{m7} value would make it hard to reduce the mitoNEET [2Fe-2S] clusters. It would be of interest to know whether pioglitazone may affect the human glutathione reductase-mediated reduction of the mitoNEET [2Fe-2S] clusters. In the experiments, mitoNEET was pre-incubated with pioglitazone before being reduced with human glutathione reductase and NADPH. As shown in Figure 2B, the mitoNEET [2Fe-2S] clusters was only partially inhibited (spectrum 5) upon binding of pioglitazone. The result is consistent with the notion that binding of pioglitazone to mitoNEET negatively shifts the E_{m7} of the mitoNEET [2Fe-2S] clusters [20, 23] and impedes reduction of the mitoNEET [2Fe-2S] clusters by glutathione reductase.

The oxidized mitoNEET [2Fe-2S] clusters have two major UV-visible absorption peaks at 458 nm and 540 nm [12]. When the mitoNEET [2Fe-2S] clusters are reduced, the absorption peak at 458 nm is changed to 420 nm [23]. Here we took advantage of the distinct UV-visible absorption peaks of the mitoNEET [2Fe-2S] clusters to explore the reduction kinetics of the mitoNEET [2Fe-2S] clusters by human glutathione reductase. When mitoNEET was incubated with a catalytic amount of human glutathione reductase in the presence of NADPH at 37°C under anaerobic conditions, the absorption peak at 458 nm of the oxidized mitoNEET [2Fe-2S] clusters was quickly decreased and replaced with the absorption peak at 420 nm of the reduced mitoNEET [2Fe-2S] clusters (Figure 3A). The amount of the reduced mitoNEET [2Fe-2S] clusters in the reaction solution was plotted as a function of incubation time. Under the experimental conditions, the mitoNEET [2Fe-2S] clusters were fully reduced in 20 min with a half reduction time of less than 5 min (Figure 3B).

3. The redox active disulfide center in human glutathione reductase is involved in reducing the mitoNEET [2Fe-2S] clusters

Human glutathione reductase catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as electron donor [30, 41]. If glutathione reductase reduces the mitoNEET [2Fe-2S] clusters at the same catalytic site where GSSG is reduced, we would expect competition between GSSG and the mitoNEET [2Fe-2S] clusters for the catalytic center in the enzyme. To test this idea, we explored the effect of GSSG on

the glutathione reductase-mediated reduction of the mitoNEET [2Fe-2S] clusters. Figure 4A shows that in the absence of GSSG, the mitoNEET [2Fe-2S] clusters (10 μ M) were fully reduced by 10 μ M NADPH in the presence of a catalytic amount of human glutathione reductase. In the presence of 10 μ M GSSG, however, the mitoNEET [2Fe-2S] clusters were reduced only after 20 μ M NADPH was added in the incubation solution (Figure 4B). Thus, GSSG may effectively block the human glutathione reductase-mediated reduction of the mitoNEET [2Fe-2S] clusters by competing for the same catalytic center in the enzyme (Figure 4C).

Unlike *E. coli* thioredoxin reductase and glutathione reductase (Figure 1C) or human glutathione reductase (Figure 2B), rat thioredoxin reductase has very little or no activity to reduce the mitoNEET [2Fe-2S] clusters (Figure 2B). One possible explanation is that rat thioredoxin reductase has an unusual selenocysteine in the catalytic center [31–33], which fails to reduce the mitoNEET [2Fe-2S] clusters. If that is the case, we would expect that modification of the redox active disulfide in the catalytic center of human glutathione reductase may inhibit the enzyme activity to reduce the mitoNEET [2Fe-2S] clusters.

N-ethylmaleimide (NEM), a potent thiol modifier, has been used to inhibit human glutathione reductase [34, 49]. Figure 4D shows that the NEM-treated human glutathione reductase failed to reduce the mitoNEET [2Fe-2S] clusters (spectrum 2). However, addition of sodium dithionite to the above reaction solution quickly restored the EPR signal at $g = 1.94$ of the mitoNEET [2Fe-2S] clusters (spectrum 3), suggesting that NEM inhibits human glutathione reductase to reduce the mitoNEET [2Fe-2S] clusters without directly modifying the mitoNEET [2Fe-2S] clusters. Thus, the redox active disulfide in the catalytic center of human glutathione reductase may be directly involved in reducing the mitoNEET [2Fe-2S] clusters.

5. The mitoNEET [2Fe-2S] clusters are fully reduced in the mouse heart cell extracts by NADPH

As human glutathione reductase can efficiently reduce the mitoNEET [2Fe-2S] clusters in an NADPH-dependent reaction (Figure 3), the mitoNEET [2Fe-2S] clusters may also be reduced in mammalian cells by glutathione reductase or similar enzymes. To test this idea, we prepared cell extracts from mouse heart as described in the Materials and Methods. Figure 5A shows that while the mouse heart cell extracts had no activity to reduce the mitoNEET [2Fe-2S] clusters (spectrum 2), addition of NADPH to the cell extracts reduced the mitoNEET [2Fe-2S] clusters (spectrum 3) with the EPR amplitude similar to that reduced with sodium dithionite (spectrum 4).

The cell extracts were then treated with the thiol modifier NEM to inactivate glutathione reductase or similar enzymes. As shown in Figure 5B, the NEM-treated cell extracts had no activity to reduce the mitoNEET [2Fe-2S] clusters in the presence of NADPH (spectrum 3). Nevertheless, addition of sodium dithionite to the NEM-treated cell extracts immediately restored the EPR signal at $g = 1.94$ of the reduced mitoNEET [2Fe-2S] clusters (spectrum 4), suggesting that NEM inhibits the cellular reductase activities without affecting on the mitoNEET [2Fe-2S] clusters in the mouse heart cell extracts.

6. Reduced mitoNEET [2Fe-2S] clusters in the cell extracts are transiently oxidized by hydrogen peroxide

To examine whether the mitoNEET [2Fe-2S] clusters may undergo redox transition in response to oxidative signals, we treated mouse heart cell extracts containing the pre-reduced mitoNEET [2Fe-2S] clusters with different amounts of hydrogen peroxide. As the concentration of hydrogen peroxide in the cell extracts was increased from 0 to 200 μM , the reduced mitoNEET [2Fe-2S] clusters were gradually oxidized (Figure 6A). About 100 μM hydrogen peroxide was sufficient to completely oxidize 5 μM mitoNEET [2Fe-2S] clusters in the cell extracts (Figure 6B). Importantly, addition of sodium dithionite to the hydrogen peroxide-treated cell extracts quickly restored the EPR signal at $g = 1.94$ of the reduced mitoNEET [2Fe-2S] clusters (Figure 6A), suggesting that hydrogen peroxide oxidizes the mitoNEET [2Fe-2S] clusters without disrupting the clusters in the protein.

We then asked whether oxidation of the mitoNEET [2Fe-2S] clusters by hydrogen peroxide is reversible in the mouse heart cell extracts. In the experiment, the mitoNEET [2Fe-2S] clusters oxidized by hydrogen peroxide in the cell extracts with excess NADPH were re-incubated at 37°C under anaerobic conditions. As shown in Figure 7, the oxidized mitoNEET [2Fe-2S] clusters in the cell extracts were almost fully re-reduced after about 30 min re-incubation.

DISCUSSION

Human mitoNEET is a small mitochondrial outer membrane protein containing a redox active [2Fe-2S] cluster. Here we find that the mitoNEET [2Fe-2S] clusters can be efficiently reduced by human glutathione reductase in an NADPH-dependent reaction. On the other hand, rat thioredoxin reductase, a homolog of human thioredoxin reductase, has very little or no activity to reduce the mitoNEET [2Fe-2S] clusters. Oxidized glutathione appears to compete for the catalytic site of human glutathione reductase and effectively inhibits the enzyme-mediated reduction of the mitoNEET [2Fe-2S] clusters, indicating a potential dynamic link between the redox state of the mitoNEET [2Fe-2S] clusters and the ratio of oxidized glutathione to reduced glutathione in cells. Additional studies further revealed that the reduced mitoNEET [2Fe-2S] clusters in the mouse heart cell extracts can be transiently oxidized by hydrogen peroxide without disruption of the clusters in the protein. The results suggest that the mitoNEET [2Fe-2S] clusters may undergo redox transition to regulate energy metabolism in mitochondria in response to oxidative signals.

The finding that human glutathione reductase [30] can reduce the mitoNEET [2Fe-2S] clusters (Figure 2B) is novel. While the underlying mechanism is not immediately clear, it seems that the redox active disulfide in the catalytic center of human glutathione reductase has a crucial role in reducing the mitoNEET [2Fe-2S] clusters. Both *E. coli* thioredoxin reductase [44] and glutathione reductase [37] contain redox active disulfide in the catalytic center and have the same activity to reduce the mitoNEET [2Fe-2S] clusters (Figure 1). In contrast, rat thioredoxin reductase, a homolog of human thioredoxin reductase that contains an unusual selenocysteine in the catalytic center [31–33], has very little or no activity to reduce the mitoNEET [2Fe-2S] clusters (Figure 2B). *E. coli* succinic semialdehyde dehydrogenase [38] and 2,4-dienoyl-CoA reductase [39], which use NADPH as substrate

but do not have a redox active disulfide in the catalytic center, also fail to reduce the mitoNEET [2Fe-2S] clusters (data not shown). Furthermore, modification of the redox active disulfide in human glutathione reductase by thiol modifier NEM [34, 49] completely inhibits the enzyme activity to reduce the mitoNEET [2Fe-2S] clusters (Figures 4 and 5). Taken together, we conclude that the redox active disulfide in the catalytic center of human glutathione reductase may be directly involved in reducing the mitoNEET [2Fe-2S] clusters (Figure 8).

While the mechanism for reduction of disulfide by iron-sulfur cluster in proteins has been extensively investigated in plant ferredoxin-thioredoxin reductase [50], the reverse reaction (reduction of iron-sulfur cluster in proteins by dithiol) is essentially unknown. Interestingly, Fridovich's group previously reported that *E. coli* thioredoxin reductase may produce superoxide by transferring a single electron to dioxygen [51]. Perhaps, *E. coli* thioredoxin reductase utilizes similar mechanism to reduce the mitoNEET [2Fe-2S] clusters (Figure 1C). More recently, Buckel's group showed that butyryl-CoA dehydrogenase from *Acidaminococcus fermentans*, a FAD-containing enzyme, can reduce the ferredoxin [4Fe-4S] cluster and crotonyl-CoA simultaneously [52] in a bifurcation reaction [53]. FAD acts as an electron gate to deliver one electron at a time for reduction of the ferredoxin [4Fe-4S] cluster and crotonyl-CoA in butyryl-CoA dehydrogenase [52]. As human glutathione reductase also contains FAD (Figure 2A), we propose that during the reaction cycle, an intermediate state of FADH⁻ coupled with the redox active disulfide formed in human glutathione reductase [30] may deliver single electrons to reduce the mitoNEET [2Fe-2S] clusters (Figure 8).

Under normal physiological conditions, the mitoNEET [2Fe-2S] clusters are mostly in reduced state *in vivo* [23] (Figure 5). However, when cells are under oxidative stress or during apoptosis and differentiation, the intracellular redox potential could increase from -325 mV (pH 7.0) [22] to as high as +200 mV [54], which would result in oxidation of the mitoNEET [2Fe-2S] clusters. The closest distance between two [2Fe-2S] clusters in the mitoNEET dimer is about 14 Å [13–15]. Thus, oxidation of the two [2Fe-2S] clusters in the mitoNEET dimer may result in conformational change of the mitoNEET dimer via electric repulsion. Since mitoNEET can potentially form protein complexes with E3 ubiquitin ligase Parkin [28], V-ATPase [29], tax-responsive enhancer element-binding protein 107 (RPL6) [29], translocase of outer mitochondrial membrane 7 homolog (TOMM7) [29], mitochondrial outer membrane import complex protein 1 (MTX1) [29], polyubiquitin-C (UBC) [55], and glutamate dehydrogenase 1 [56], we postulate that mitoNEET may modulate the functions of its binding partners in mitochondria via redox transition of its [2Fe-2S] clusters in response to redox signals.

MitoNEET-like proteins are widely distributed in the three domains of life [57–59]. In humans, there are two mitoNEET-like proteins: Miner1 [60] and Miner2 [5]. Like mitoNEET, the C-terminal domain of Miner1 hosts a redox active [2Fe-2S] cluster via three cysteine and one histidine residues [60]. Mutations in Miner1 have been attributed to Wolfram Syndrome 2, a disease characterized by juvenile onset diabetes mellitus and optic atrophy [61]. Miner1 was initially localized on endoplasmic reticulum [61], but recent studies indicated that Miner1 also localizes on mitochondrial outer membrane [62, 63] and

has a crucial role in regulating sulfhydryl redox status, the unfold protein response, and Ca^{2+} homeostasis in mitochondria via a yet unknown regulatory mechanism [63]. While the physiological function of Miner2 remains unknown, Miner2 is also a mitochondrial outer membrane protein [5] hosting two [2Fe-2S] clusters per monomer (unpublished results). It may be envisioned that human mitoNEET, Miner1 and Miner2 may modulate different functions in mitochondria via redox transition of the [2Fe-2S] clusters.

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List of Abbreviations

E_m7	redox midpoint potential at pH 7.0
EPR	electron paramagnetic resonance
eGor	<i>E. coli</i> glutathione reductase
eTrxB	<i>E. coli</i> glutathione reductase
GSSG	oxidized glutathione
hGSR	human glutathione reductase mito, human mitoNEET
NEM	N-ethylmaleimide
TZD	thiazolidinedione

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Figure 1. The mitoNEET [2Fe-2S] clusters are reduced by *E. coli* thioredoxin reductase and glutathione reductase

A) EPR spectra of the mitoNEET [2Fe-2S] clusters in the *E. coli* cell extracts. Purified mitoNEET (10 μ M of [2Fe-2S] clusters) (spectrum 1) was incubated with NADPH (50 μ M) (spectrum 2), the *E. coli* cell extracts (4 mg/ml of total protein) (spectrum 3), or NADPH (50 μ M) and the *E. coli* cell extracts (4 mg/ml of total protein) (spectrum 4) at 37°C for 20 min under anaerobic conditions. Spectrum 5, purified mitoNEET (10 μ M of [2Fe-2S] clusters) reduced with 2 mM sodium dithionite. The EPR signal at $g = 1.94$ represents the reduced mitoNEET [2Fe-2S] clusters. **B)** purified *E. coli* thioredoxin reductase and glutathione reductase. Spectrum 1, UV-visible absorption spectra of purified *E. coli* thioredoxin

reductase (eTrxB). Spectrum 2, UV-visible absorption spectra of purified glutathione reductase (eGor). eTrxB and eGor shown in B) contained about 15 μM of FAD. Insert is a photograph of the SDS-PAGE of purified *E. coli* thioredoxin reductase (lane 1) and glutathione reductase (lane 2). C), reduction of the mitoNEET [2Fe-2S] clusters by *E. coli* thioredoxin reductase and glutathione reductase. Purified mitoNEET (10 μM of [2Fe-2S] clusters) (spectrum 1) was incubated with NADPH (50 μM) (spectrum 2), *E. coli* thioredoxin reductase (eTrxB) (1 μM) (spectrum 3), *E. coli* glutathione reductase (eGor) (1 μM) (spectrum 4), *E. coli* thioredoxin reductase (1 μM) and NADPH (50 μM) (spectrum 5), or *E. coli* glutathione reductase (1 μM) and NADPH (50 μM) (spectrum 6) at 37°C for 20 min under anaerobic conditions. The EPR signal at $g_{av} = 1.94$ represents the reduced mitoNEET [2Fe-2S] clusters. The results are the representatives of three independent experiments.

Figure 2. Reduction of the mitoNEET [2Fe-2S] clusters by human glutathione reductase
A) UV-visible absorption spectra of purified *E. coli* glutathione reductase (eGor) and human glutathione reductase (hGSR). Each protein shown in A) contained about 15 μ M of FAD. **B)** reduction of the mitoNEET [2Fe-2S] clusters by human cytosolic glutathione reductase. A mix of purified mitoNEET (10 μ M of [2Fe-2S] clusters) and NADPH (100 μ M) (spectrum 1) was incubated with rat thioredoxin reductase (0.5 μ M) (spectrum 2), *E. coli* glutathione reductase (eGor) (0.5 μ M) (spectrum 3), human glutathione reductase (hGSR) (0.5 μ M) (spectrum 4) at 37°C for 20 min under anaerobic conditions. Spectrum 5, a mix of mitoNEET (10 μ M of [2Fe-2S] clusters) and NADPH (100 μ M) was pre-incubated with pioglitazone (2.5 mM) at room temperature for 30 min before being reduced with human glutathione reductase (0.5 μ M). The results are the representatives of three independent experiments.

Figure 3. Reduction kinetics of the mitoNEET [2Fe-2S] clusters by human glutathione reductase (hGSR)

A), reduction of (the mitoNEET [2Fe-2S] clusters by human glutathione reductase. Purified mitoNEET (10 μ M of [2Fe-2S] clusters) was incubated with hGSR (1 μ M) and NADPH (20 μ M) at 37°C under anaerobic conditions. Spectra were taken at 0, 2, 5, 10, 20, and 30 min after hGSR was added to the reaction solution. The absorption peak at 458 nm represents the oxidized mitoNEET [2Fe-2S] clusters, and the peak at 420 nm represents the reduced mitoNEET [2Fe-2S] clusters. **B)**, reduction kinetics of the mitoNEET [2Fe-2S] clusters by hGSR. The spectra in A) were deconvoluted to the reduced and oxidized mitoNEET [2Fe-2S] clusters using KaleidaGraph. The percentage of the oxidized mitoNEET [2Fe-2S] clusters was plotted as a function of incubation time. The results are the representatives of three independent experiments.

Figure 4. The redox active disulfide in human glutathione reductase is crucial for reducing the mitoNEET [2Fe-2S] clusters

A), reduction of the mitoNEET [2Fe-2S] clusters by human glutathione reductase without the oxidized glutathione. Purified mitoNEET (10 μM of [2Fe-2S] clusters) was incubated with 0, 5, 10, and 20 μM NADPH and hGSR (0.5 μM) at 37°C for 20 min under anaerobic conditions. The samples were subjected to the UV-Visible absorption measurements. **B)**, same as in A) except 10 μM GSSG was incubated in each reaction solution. **C)**, effect of GSSG on reduction of the mitoNEET [2Fe-2S] clusters. The amount of the reduced

mitoNEET [2Fe-2S] clusters was plotted as a function of NADPH in the incubation solution. Closed circles, no GSSG. Closed squares, with 10 μ M GSSG. **D**), inhibition of human glutathione reductase by NEM (N-ethylmaleimide). Purified hGSR was pre-incubated with or without NEM (2 mM) at room temperature for 3 hours. MitoNEET was then incubated with NADPH (20 μ M) and hGSR (0.5 μ M) (spectrum 1) or NADPH (20 μ M) and the NEM-treated hGSR (0.5 μ M) (spectrum 2) at 37°C for 20 min under anaerobic conditions. Spectrum 3, mitoNEET incubated with the NEM-treated hGSR and NADPH was reduced with sodium dithionite (2 mM). The results are the representatives of three independent experiments.

Figure 5. The mitoNEET [2Fe-2S] clusters are reduced in the mouse heart cell extracts by NADPH

A), reduction of the mitoNEET [2Fe-2S] clusters in the mouse heart cell extracts. Purified mitoNEET (10 μ M of [2Fe-2S] clusters) (spectrum 1) was incubated with the mouse heart cell extracts (2 mg/ml of total protein) (spectrum 2), or the mouse heart cell extracts supplemented with NADPH (100 μ M) (spectrum 3) at 37°C for 20 min under anaerobic conditions. Spectrum 4, mitoNEET mixed with the mouse heart cell extracts was treated with sodium dithionite (2 mM) under anaerobic conditions. Spectrum 5, the mouse heart cell extracts were incubated with NADPH without exogenous human mitoNEET. The EPR signal at $g = 1.94$ represents the reduced mitoNEET [2Fe-2S] clusters. **B)**, inhibition of the NADPH-mediated reduction of the mitoNEET [2Fe-2S] clusters by N-ethylmaleimide (NEM). The mouse heart cell extracts were pre-treated with 0 mM (spectrum 2), and 2 mM (spectrum 3) NEM at room temperature for 3 hours. Purified mitoNEET (10 μ M [2Fe-2S] clusters) mixed with NADPH (100 μ M) (spectrum 1) was then incubated with the NEM-pretreated cell extracts for 20 min at 37°C under anaerobic conditions. Spectrum 4, mitoNEET after incubation with the NEM-pretreated cell extracts and NADPH was reduced with sodium dithionite (2 mM). The results are the representative of at least three independent experiments.

Figure 6. Hydrogen peroxide-mediated oxidation of the mitoNEET [2Fe-2S] clusters in the cell extracts

A) oxidation of the mitoNEET [2Fe-2S] clusters in the cell extracts by H_2O_2 . Purified mitoNEET (5 μM of [2Fe-2S] clusters) in the mouse heart cell extracts (1 mg/mL of total protein) pre-reduced with NADPH (100 μM) was treated with 0 μM (spectrum 1), 20 μM (spectrum 2), 50 μM (spectrum 3), 100 μM (spectrum 4), and 200 μM (spectrum 5) H_2O_2 for 5 min. Spectrum 6, the mitoNEET treated with 200 μM H_2O_2 was immediately reduced with sodium dithionite (2 mM). **B)** titration of hydrogen peroxide in the incubation solution. The amplitudes of the EPR signal at $g = 1.94$ of the reduced mitoNEET [2Fe-2S] clusters were plotted as a function of the H_2O_2 concentration in the cell extracts. The results are the representative from three independent experiments.

Figure 7. Re-reduction of the mitoNEET [2Fe-2S] clusters in the cell extracts after hydrogen peroxide exposure

A) re-reduction of the mitoNEET [2Fe-2S] clusters in the cell extracts. Purified mitoNEET (5 μ M of [2Fe-2S] clusters) was incubated with NADPH (2 mM) and the mouse heart cell extracts (1 mg/mL of total protein) at 37°C for 15 min under anaerobic conditions (spectrum 1). The pre-reduced mitoNEET [2Fe-2S] clusters in the cell extracts were treated with 100 μ M hydrogen peroxide for 5 min (spectrum 2), and re-incubated at 37°C under anaerobic conditions for 10 min (spectrum 3), 20 min (spectrum 4), and 30 min (spectrum 5). **B)** time course of re-reduction of the mitoNEET [2Fe-2S] clusters in the cell extracts by NADPH after the hydrogen peroxide treatment. The amplitudes of the EPR signal at $g = 1.94$ were plotted as a function of time after addition of H_2O_2 to the incubation solution. The results are the representatives of three independent experiments.

Figure 8. A proposed model for redox transition of the mitoNEET [2Fe-2S] clusters in response to hydrogen peroxide in mammalian cells

Cytosolic reductases (e.g. glutathione reductase) transfer electrons from NADPH to FAD to form FADH⁻ which in turn transfers single electron to reduce the mitoNEET [2Fe-2S] clusters. When cells are subjected to oxidative stress, the reduced mitoNEET [2Fe-2S] clusters are reversibly oxidized by hydrogen peroxide.