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Aaron P. Landry  
*Louisiana State University*

Huangen Ding  
*Louisiana State University*

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# Redox Control of Human Mitochondrial Outer Membrane Protein MitoNEET [2Fe-2S] Clusters by Biological Thiols and Hydrogen Peroxide\*

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Aaron P. Landry and Huanggen Ding<sup>1</sup>

From the Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

**Background:** MitoNEET is a target of the type II diabetes drug pioglitazone and contains a [2Fe-2S] cluster.

**Results:** The mitoNEET [2Fe-2S] cluster can be reduced by biological thiols and reversibly oxidized by hydrogen peroxide.

**Conclusion:** The redox state of mitoNEET [2Fe-2S] clusters can be regulated by thiols and oxidative signals.

**Significance:** MitoNEET may act as a redox sensor to modulate mitochondrial functions.

The human mitochondrial outer membrane protein mitoNEET is a novel target of the type II diabetes drug pioglitazone. The C-terminal cytosolic domain of mitoNEET hosts a redox-active [2Fe-2S] cluster via an unusual ligand arrangement of three cysteine residues and one histidine residue. Here we report that human mitoNEET [2Fe-2S] clusters are fully reduced when expressed in *Escherichia coli* cells. *In vitro* studies show that purified mitoNEET [2Fe-2S] clusters can be partially reduced by monothiols such as reduced glutathione, L-cysteine or N-acetyl-L-cysteine and fully reduced by dithiothreitol or the *E. coli* thioredoxin/thioredoxin reductase system under anaerobic conditions. Importantly, thiol-reduced mitoNEET [2Fe-2S] clusters can be reversibly oxidized by hydrogen peroxide without disruption of the clusters *in vitro* and in *E. coli* cells, indicating that mitoNEET may act as a sensor of oxidative signals to regulate mitochondrial functions via its [2Fe-2S] clusters. Furthermore, the binding of the type II diabetes drug pioglitazone in mitoNEET effectively inhibits the thiol-mediated reduction of [2Fe-2S] clusters, suggesting that pioglitazone may modulate the function of mitoNEET by blocking the thiol-mediated reduction of [2Fe-2S] clusters in the protein.

The human mitochondrial protein mitoNEET is a novel target of the type II diabetes drugs thiazolidinediones, such as pioglitazone (1). Genetic studies have shown that the deletion of mitoNEET in mice results in a reduced oxidative phosphorylation capacity in mitochondria (2). Although an increased expression of mitoNEET in adipocytes in mice enhances lipid uptake and storage and inhibits mitochondrial iron transport into the matrix, depletion of mitoNEET in adipocytes leads to less weight gain in mice (3), suggesting that mitoNEET may regulate the energy metabolism in mitochondria (1). Recent studies further indicated that mitoNEET may have a central role in neurodegenerative diseases (4, 5) and breast cancer pro-

liferation (6) by maintaining mitochondrial homeostasis in cells.

MitoNEET localizes on mitochondrial outer membranes (2) via the N-terminal transmembrane  $\alpha$ -helix (residues 14–32) (1). Expression of the soluble C-terminal domain (residues 33–108) of mitoNEET in *Escherichia coli* cells produced a protein that contains a [2Fe-2S] cluster (7). Crystallographic studies revealed that mitoNEET exists as a homodimer, with each monomer hosting a [2Fe-2S] cluster via an unusual ligand arrangement of three cysteine residues (Cys-72, Cys-74, and Cys-83) and one histidine residue (His-87) (8–10). The [2Fe-2S] clusters in mitoNEET are redox-active, with a midpoint redox potential at pH 7 ( $E_{m7}$ ) of  $\sim 0$  mV (11, 12). Substitution of the unique His-87 with cysteine in mitoNEET shifts the  $E_{m7}$  value of the [2Fe-2S] clusters from 0 to  $-320$  mV (11) and increases the stability of the cluster in the protein (13). The redox property and stability of the [2Fe-2S] clusters in mitoNEET are also modulated by the type II diabetes drug pioglitazone (11), excess zinc (14), resveratrol-3-sulfate (15), NADPH (16), the interdomain interactions (17, 18), and the hydrogen bond network in the protein (19), suggesting that mitoNEET may act as a sensor of multiple signals to regulate mitochondrial functions (19). Interestingly, purified mitoNEET can also transfer the [2Fe-2S] clusters to apo-protein with a 50% completion time of about 120 min at room temperature (20, 21). Because mitochondria are the primary sites for iron-sulfur cluster biogenesis (22), it is appealing to consider that mitoNEET may participate in the iron-sulfur cluster biogenesis process in human cells (20, 21). Nevertheless, the iron-sulfur cluster transfer occurs only when the [2Fe-2S] clusters in mitoNEET are oxidized (20) and the cluster transfer process is inhibited by NADPH (21), suggesting that the mitoNEET-mediated iron-sulfur cluster transfer could be regulated by the redox state of the [2Fe-2S] clusters and by intracellular NADPH.

Because the cytosolic redox potential in eukaryotic cells is estimated to be around  $-325$  mV (pH 7.0) (23), it is expected that the mitoNEET [2Fe-2S] clusters ( $E_{m7} = 0$  mV) (11, 12) would be in a reduced state in cells under physiological conditions. Nevertheless, the redox state of the mitoNEET [2Fe-2S] clusters in cells has not been demonstrated, and specific cellular components that may reduce the mitoNEET [2Fe-2S] clusters

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<sup>1</sup> To whom correspondence should be addressed: Dept. of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803. E-mail: hding@lsu.edu.

## Reduction of mitoNEET [2Fe-2S] Clusters

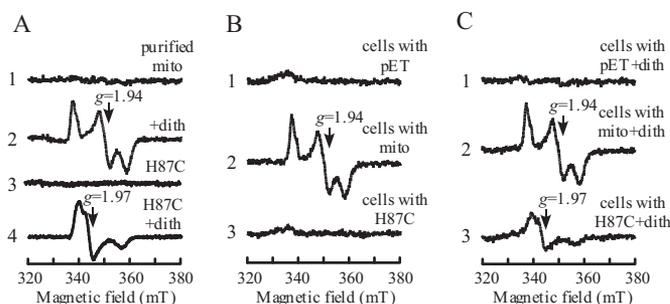
have not been identified. Here we report that human mitoNEET [2Fe-2S] clusters are in the fully reduced state when expressed in *E. coli* cells under normal growth conditions and that purified mitoNEET [2Fe-2S] clusters can be partially reduced by monothiois such as reduced glutathione, L-cysteine, and N-acetyl-L-cysteine and fully reduced by dithiothreitol and the *E. coli* thioredoxin-1 reduced by thioredoxin reductase and NADPH. Importantly, the reduced mitoNEET [2Fe-2S] clusters can be reversibly oxidized by hydrogen peroxide without disruption of the clusters, indicating that the redox state of the mitoNEET [2Fe-2S] clusters can be modulated by biological thiols and oxidative signals. Furthermore, we find that the type II diabetes drug pioglitazone can effectively inhibit the thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters *in vitro*, suggesting that pioglitazone may modulate the function of mitoNEET by blocking the thiol-mediated reduction of the [2Fe-2S] clusters in the protein.

### EXPERIMENTAL PROCEDURES

**Protein Purification**—The gene encoding human mitoNEET<sub>33–108</sub> (containing amino acid residues 33–108) was cloned previously from the human cDNA library as described in Ref. 14. The mitoNEET mutant in which histidine 87 was substituted with cysteine (H87C) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene). The constructed mutation was confirmed by direct sequencing (Operon Co.). Human mitoNEET and the mitoNEET mutant proteins were prepared following procedures described previously (14). *E. coli* thioredoxin-1 (TrxA) and thioredoxin reductase (TrxB) were produced from *E. coli* cells using the expression vectors pDL59 (24) and pTrR301 (25), respectively, and purified as described in Ref. 26. Both *E. coli* thioredoxin and thioredoxin reductase were purified as the native form. 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 concentration of purified mitoNEET or the mitoNEET mutant H87C was measured at 280 nm using an extinction coefficient of 8.6 mM<sup>-1</sup>cm<sup>-1</sup>. The protein concentration of thioredoxin-1 and thioredoxin reductase was determined at 280 nm using extinction coefficients of 14.2 and 17.7 mM<sup>-1</sup>cm<sup>-1</sup>, respectively. A Beckman DU640 UV-visible spectrometer equipped with a temperature control was used for measuring absorption spectra.

**Other Chemicals**—Pioglitazone, L-cysteine, N-acetyl-L-cysteine, reduced glutathione, and other chemicals were purchased from Sigma. Isopropyl β-D-1-thiogalactopyranoside, NADPH, kanamycin, ampicillin, and dithiothreitol were from Research Product International Co. Pioglitazone was dissolved in dimethyl sulfoxide as a stock solution containing 25 mM pioglitazone. An equal amount of dimethyl sulfoxide was added to the samples as a control.

**EPR Measurements**—For whole-cell EPR measurements, *E. coli* cells were grown for 2 h before the recombinant protein expression was induced. After 2 h of protein expression, cells were harvested, washed once with phosphate buffer (pH 7.5), and resuspended in phosphate buffer or fresh LB medium. An aliquot (350 μl) of the *E. coli* cells was then transferred to the EPR tube and frozen immediately in liquid nitrogen. For the



**FIGURE 1. MitoNEET [2Fe-2S] clusters are fully reduced in *E. coli* cells.** A, EPR spectra of purified mitoNEET (mito) [2Fe-2S] clusters. Purified mitoNEET and mitoNEET mutant H87C proteins (20 μM [2Fe-2S] clusters) were reduced with sodium dithionite (dith) (2 mM). Spectrum 1 shows wild-type mitoNEET [2Fe-2S] clusters. Spectrum 2 shows wild-type mitoNEET [2Fe-2S] clusters reduced with dithionite. Spectrum 3 shows mitoNEET mutant H87C [2Fe-2S] clusters. Spectrum 4 shows mitoNEET mutant H87C [2Fe-2S] clusters reduced with dithionite. mT, millitesla. B, EPR spectra of *E. coli* cells with or without recombinant mitoNEET. Recombinant proteins were expressed in *E. coli* cells for 2 h at room temperature, and then cells were concentrated to an  $A_{600\text{ nm}}$  of 15. Spectrum 1 shows *E. coli* cells containing the expression plasmid pET28b+ (pET) only. Spectrum 2 shows *E. coli* cells expressing wild-type mitoNEET. Spectrum 3 shows *E. coli* cells expressing the mitoNEET mutant H87C. C, EPR spectra of *E. coli* cells after reduction with sodium dithionite (2 mM). Spectrum 1 shows *E. coli* cells containing the expression plasmid pET28b+ only. Spectrum 2 shows *E. coli* cells expressing wild-type mitoNEET. Spectrum 3 shows *E. coli* cells expressing the mitoNEET mutant H87C. The results are representative of three independent experiments.

reduced samples, freshly prepared sodium dithionite was added to the *E. coli* cells before the EPR samples were prepared. For protein sample preparation, purified mitoNEET [2Fe-2S] clusters dissolved in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl were purged with pure argon gas in sealed vials for 15 min before reductant was added under anaerobic conditions. After incubation at 37 °C for 20 min, samples were transferred to the EPR tubes and frozen immediately in liquid nitrogen. 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 as follows: microwave frequency, 9.47 GHz; microwave power, 10.0 milliwatt; modulation frequency, 100 kHz; modulation amplitude, 1.2 millitesla; temperature, 20 K; receive gain, 10<sup>5</sup>.

### RESULTS

**Human MitoNEET [2Fe-2S] Clusters Expressed in *E. coli* Cells Are Fully Reduced**—Purified human mitoNEET [2Fe-2S] clusters are in an oxidized state and have no EPR signals (Fig. 1A). When purified mitoNEET [2Fe-2S] clusters are reduced with sodium dithionite ( $E_{m7} = -600$  mV (27)), a rhombic EPR spectrum with  $g_x = 1.895$ ,  $g_y = 1.937$ , and  $g_z = 2.005$  appears (Fig. 1A), as reported previously (7, 19, 28, 29). The  $g_x$ ,  $g_y$ , and  $g_z$  values represent the anisotropic g-factors of a typical [2Fe-2S] cluster in proteins. In parallel, we also prepared the mitoNEET mutant H87C, in which the unique ligand His-87 of the [2Fe-2S] cluster is replaced with Cys. Although purified mitoNEET H87C has no EPR signal, addition of sodium dithionite produces a new rhombic EPR spectrum of the [2Fe-2S] cluster, with  $g_x = 1.896$ ,  $g_y = 1.970$ , and  $g_z = 1.992$ , that is distinct from that of the wild-type mitoNEET (Fig. 1A).

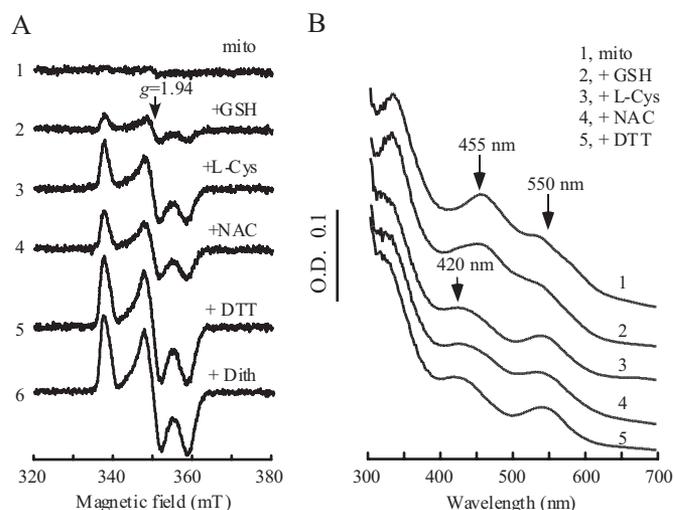
The EPR measurement provides an efficient and non-invasive approach to determine the redox state of iron-sulfur clusters in proteins in solutions or in whole cells (30, 31). Here, we

took the advantage of EPR to explore the redox state of the mitoNEET [2Fe-2S] clusters expressed in *E. coli* cells. Fig. 1B shows that *E. coli* cells without any recombinant proteins have very little or no EPR signals because of low concentrations of endogenous iron-sulfur proteins in cells (30, 31). Under the same experimental conditions, *E. coli* cells expressing human mitoNEET show a rhombic EPR signal at  $g = 1.94$ , which is identical to that of the reduced mitoNEET [2Fe-2S] clusters (Fig. 1A). In contrast, *E. coli* cells expressing the mitoNEET mutant H87C do not have any EPR signals (Fig. 1B), although both mitoNEET and the mitoNEET mutant H87C are expressed similarly in *E. coli* cells on the basis of SDS-PAGE gel analysis (data not shown). This is likely because the mutation of His-87 to Cys shifts the  $E_{m7}$  value of the mitoNEET [2Fe-2S] clusters from 0 to  $-320$  mV (11). Because the cytosolic redox potential of *E. coli* cells is about  $-260$  mV (32), the mitoNEET mutant H87C [2Fe-2S] clusters would be mostly in an oxidized (EPR-silent) state in *E. coli* cells.

To further examine the redox state of the [2Fe-2S] clusters in wild-type mitoNEET and the mitoNEET mutant H87C expressed in *E. coli* cells, excess sodium dithionite is added to the cells to reduce all iron-sulfur proteins. Fig. 1C shows that addition of sodium dithionite to *E. coli* cells without recombinant proteins does not produce any new EPR signals. On the other hand, addition of sodium dithionite to *E. coli* cells expressing the mitoNEET mutant H87C generates an EPR signal at  $g = 1.97$ , which is the same as that of the reduced mitoNEET mutant H87C [2Fe-2S] clusters (Fig. 1A). Thus, the oxidized [2Fe-2S] clusters in the mitoNEET mutant H87C in *E. coli* cells can be reduced by sodium dithionite. In contrast, the EPR signal at  $g = 1.94$  of the *E. coli* cells expressing the wild-type mitoNEET is not changed by addition of sodium dithionite, suggesting that the wild-type mitoNEET [2Fe-2S] clusters are fully reduced in *E. coli* cells even before the addition of sodium dithionite. Because the cytosolic redox potential in eukaryotic cells ( $-325$  mV (23)) is about 65 mV lower than that in *E. coli* cells ( $-260$  mV (32)), we postulate that the mitoNEET [2Fe-2S] clusters are most likely in a reduced state in human cells under physiological conditions.

**Human MitoNEET [2Fe-2S] Clusters Can Be Reduced by Biological Thiols**—Although it has been shown that the mitoNEET [2Fe-2S] clusters are redox-active (11, 12), specific biological molecules that can reduce or oxidize the mitoNEET [2Fe-2S] clusters are unknown. Because the mitoNEET [2Fe-2S] clusters are fully reduced in *E. coli* cells, we reason that the mitoNEET [2Fe-2S] clusters may be reduced by cellular reducing components that are common in both human and *E. coli* cells. However, NADPH is not one of them because NADPH fails to reduce the mitoNEET [2Fe-2S] clusters *in vitro* (21). Among other cellular reducing components, biological thiols are abundant. More importantly, biological thiols are able to form relatively stable intermediate thiol free radicals (33, 34) that can potentially provide electrons for the single-electron reduction of the oxidized [2Fe-2S] clusters in mitoNEET.

To test this idea, purified mitoNEET was incubated with thiols under anaerobic conditions. After incubation, the samples were subjected to EPR and UV-visible absorption measurements. The reduced mitoNEET [2Fe-2S] clusters were observed from the

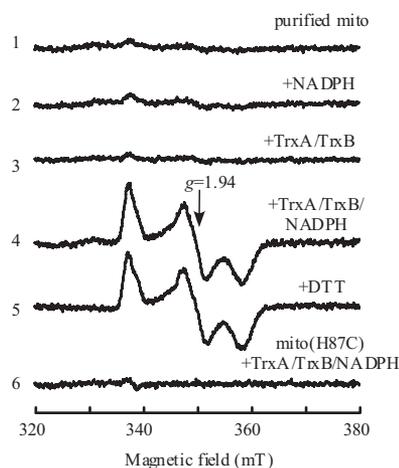


**FIGURE 2. MitoNEET [2Fe-2S] clusters can be reduced by biological thiols *in vitro*.** A, EPR spectra of the mitoNEET [2Fe-2S] clusters after reduction with different thiols. Purified mitoNEET [2Fe-2S] clusters ( $14 \mu\text{M}$ ) (spectrum 1) were incubated with 2 mM of GSH (spectrum 2), L-cysteine (L-Cys) (spectrum 3), N-acetyl-L-cysteine (NAC) (spectrum 4), or DTT (dith) (spectrum 5) at  $37^\circ\text{C}$  for 20 min under anaerobic conditions. The EPR spectrum of fully reduced mitoNEET [2Fe-2S] clusters (spectrum 6) was obtained by adding sodium dithionite (2 mM). mT, millitesla. B, UV-visible spectra of mitoNEET [2Fe-2S] clusters after incubation with biological thiols. Purified mitoNEET [2Fe-2S] clusters ( $14 \mu\text{M}$ ) (spectrum 1) was incubated with 2 mM of GSH (spectrum 2), L-cysteine (spectrum 3), N-acetyl-L-cysteine (spectrum 4), or DTT (spectrum 5) at  $37^\circ\text{C}$  for 20 min under anaerobic conditions. The absorption peaks at 455 nm and 550 nm represent the oxidized mitoNEET [2Fe-2S] clusters, and the new absorption peak at 420 nm represents the reduced mitoNEET [2Fe-2S] clusters.

EPR signal at  $g = 1.94$ , and the UV-visible absorption peaks at 420 and 550 nm. As shown in Fig. 2, all biological thiols tested (reduced glutathione, L-cysteine, and N-acetyl-L-cysteine) are able to partially reduce the mitoNEET [2Fe-2S] clusters. Reduced glutathione appears to have the least ability to reduce the mitoNEET [2Fe-2S] clusters. L-cysteine and N-acetyl-L-cysteine are able to reduce about 50% of the mitoNEET [2Fe-2S] clusters in solution under anaerobic conditions, in comparison with the fully reduced mitoNEET [2Fe-2S] clusters by sodium dithionite. A further increase of L-cysteine concentration (up to 50-fold excess over the mitoNEET [2Fe-2S] clusters) or incubation time (up to 60 min) does not increase the amount of the reduced mitoNEET [2Fe-2S] clusters in the incubation solution (data not shown), suggesting that L-cysteine could not fully reduce the mitoNEET [2Fe-2S] clusters under the experimental conditions. Interestingly, incubation with dithiothreitol, a dithiol that has an  $E_{m7}$  value of  $-323$  mV (35), almost fully reduced the mitoNEET [2Fe-2S] clusters under the same experimental conditions (Fig. 2).

**Human MitoNEET [2Fe-2S] Clusters Can Be Fully Reduced by the *E. coli* Thioredoxin/Thioredoxin Reductase System**—The biological equivalent of dithiothreitol is the ubiquitous thioredoxin (36). The  $E_{m7}$  value of *E. coli* thioredoxin-1 is about  $-270$  mV (37), which is close to that of dithiothreitol (35). To test whether the oxidized mitoNEET [2Fe-2S] clusters can be reduced by thioredoxin-1, we prepared *E. coli* thioredoxin-1 and thioredoxin reductase as described previously (26). When purified mitoNEET [2Fe-2S] clusters are incubated with equimolar concentrations of the *E. coli* thioredoxin/thioredoxin reductase or an excess amount of NADPH under anaerobic

## Reduction of mitoNEET [2Fe-2S] Clusters



**FIGURE 3. Reduction of mitoNEET [2Fe-2S] clusters by the thioredoxin reductase system *in vitro*.** Purified mitoNEET (mito) [2Fe-2S] clusters (10  $\mu\text{M}$ ) (spectrum 1) were incubated with NADPH (500  $\mu\text{M}$ ) (spectrum 2); *E. coli* thioredoxin (*TrxA*, 10  $\mu\text{M}$ ) and thioredoxin reductase (*TrxB*, 10  $\mu\text{M}$ ) (spectrum 3); or *E. coli* thioredoxin (10  $\mu\text{M}$ ), thioredoxin reductase (10  $\mu\text{M}$ ), and NADPH (500  $\mu\text{M}$ ) (spectrum 4) at 37 °C for 20 min under anaerobic conditions. Spectrum 5 shows purified mitoNEET [2Fe-2S] clusters reduced with dithiothreitol (2 mM). Spectrum 6 shows the mitoNEET mutant H87C [2Fe-2S] clusters (10  $\mu\text{M}$ ) incubated with *E. coli* thioredoxin-1 (10  $\mu\text{M}$ ), thioredoxin reductase (10  $\mu\text{M}$ ), and NADPH (500  $\mu\text{M}$ ) at 37 °C for 20 min under anaerobic conditions. After incubation, the samples were transferred to EPR tubes for EPR measurements. The results are representative of three independent experiments. *mT*, millitesla.

obic conditions, the mitoNEET [2Fe-2S] clusters remained in an oxidized state (Fig. 3). This could be because purified thioredoxin-1 is mostly oxidized during the protein purification process. Indeed, when purified mitoNEET [2Fe-2S] clusters were incubated with thioredoxin-1 prerduced with thioredoxin reductase and NADPH, the mitoNEET [2Fe-2S] clusters were fully reduced (Fig. 3). On the other hand, the mitoNEET mutant H87C [2Fe-2S] clusters that have an  $E_{m7}$  of  $-320$  mV (11) cannot be reduced by the *E. coli* thioredoxin/thioredoxin reductase system under the same experimental conditions (Fig. 3). This is consistent with the observation that the mitoNEET mutant H87C [2Fe-2S] clusters are in an oxidized state when expressed in *E. coli* cells (Fig. 1B). Taken together, the results suggest that the wild-type mitoNEET [2Fe-2S] clusters can be fully reduced by the *E. coli* thioredoxin/thioredoxin reductase system *in vitro*.

**Reduced mitoNEET [2Fe-2S] Clusters Can Be Reversibly Oxidized by Hydrogen Peroxide**—Previous studies have shown that iron-sulfur clusters in regulatory proteins can undergo redox transition in response to oxidative signals without disruption of the clusters (38–41). To explore whether the mitoNEET [2Fe-2S] clusters may also act as a redox sensor to oxidative signals, we examined the stability of the mitoNEET [2Fe-2S] clusters under oxidative stress conditions. Fig. 4A shows that, when purified mitoNEET [2Fe-2S] clusters were incubated with increasing concentrations of hydrogen peroxide (up to 50-fold excess), the UV-visible absorption spectrum of the mitoNEET [2Fe-2S] clusters remains essentially unchanged, indicating that the mitoNEET [2Fe-2S] clusters are stable in the presence of hydrogen peroxide.

We then asked whether the prerduced mitoNEET [2Fe-2S] clusters can be oxidized by hydrogen peroxide. To avoid possible effects of NADPH on the stability of the mitoNEET [2Fe-2S]

clusters (16), we prerduced the mitoNEET [2Fe-2S] clusters with excess dithiothreitol under anaerobic conditions. As shown in Fig. 4B, the prerduced mitoNEET [2Fe-2S] clusters were quickly oxidized upon addition of hydrogen peroxide. Further incubation with dithiothreitol after hydrogen peroxide treatment led to a full rereduction of the oxidized mitoNEET [2Fe-2S] clusters within about 14 min (Fig. 4, B and C). The EPR measurements of the protein samples taken from the reaction solution further confirm that the amount of the mitoNEET [2Fe-2S] clusters remain the same before and after hydrogen peroxide treatment (Fig. 4D), suggesting that the mitoNEET [2Fe-2S] clusters can undergo redox transition in response to hydrogen peroxide without disruption of the cluster.

To further explore whether the mitoNEET [2Fe-2S] clusters could undergo redox transition *in vivo* in response to hydrogen peroxide, *E. coli* cells expressing mitoNEET [2Fe-2S] clusters are treated with hydrogen peroxide. As shown in Fig. 5, addition of hydrogen peroxide (1 mM) to the *E. coli* cells largely eliminates the EPR signal at  $g = 1.94$  of the reduced mitoNEET [2Fe-2S] clusters. Reincubation of the *E. coli* cells in LB medium after hydrogen peroxide treatment quickly restores the EPR signal at  $g = 1.94$  of the reduced mitoNEET [2Fe-2S] clusters, demonstrating that the mitoNEET [2Fe-2S] clusters can undergo redox transition in response to hydrogen peroxide in *E. coli* cells.

**The Type II Diabetes Drug Pioglitazone Inhibits the Thiol-mediated Reduction of the mitoNEET [2Fe-2S] Clusters**—It has been reported that binding of the type II diabetes drug pioglitazone shifts the  $E_{m7}$  value of the mitoNEET [2Fe-2S] clusters by  $-100$  mV (11). The negative shift of the  $E_{m7}$  value would make it more difficult to reduce the mitoNEET [2Fe-2S] clusters. Nevertheless, how the redox state of the mitoNEET [2Fe-2S] clusters may be modulated by pioglitazone is unknown. With the finding that the mitoNEET [2Fe-2S] clusters can be reduced by biological thiols, we sought to investigate the effect of pioglitazone on the thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters. Purified mitoNEET is preincubated with pioglitazone, followed by reduction with thiols under anaerobic conditions. Fig. 6A shows that binding of pioglitazone had little or no effect on the dithionite-mediated reduction of the mitoNEET [2Fe-2S] clusters because dithionite is a strong reductant ( $E_{m7} = -660$  mV (27)). On the other hand, binding of pioglitazone largely inhibits the dithiothreitol-mediated reduction of the mitoNEET [2Fe-2S] clusters (Fig. 6B). Titration experiments showed that, as the concentration of pioglitazone increased gradually in the incubation solutions (from 0 to 2.5 mM), the amount of the dithiothreitol-reduced mitoNEET [2Fe-2S] clusters was decreased progressively (Fig. 6C). We also tested whether pioglitazone affected the thioredoxin-mediated reduction of the mitoNEET [2Fe-2S] clusters *in vitro*. As shown in Fig. 6D, binding of pioglitazone greatly inhibited the thioredoxin-mediated reduction of the mitoNEET [2Fe-2S] clusters, suggesting that pioglitazone can effectively block the biological thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters in cells. Nevertheless, attempts to modulate the redox state of the mitoNEET [2Fe-2S] clusters expressed in *E. coli* cells with pioglitazone were not successful. This is likely because pioglitazone may not easily penetrate bacterial cell walls and/or cell

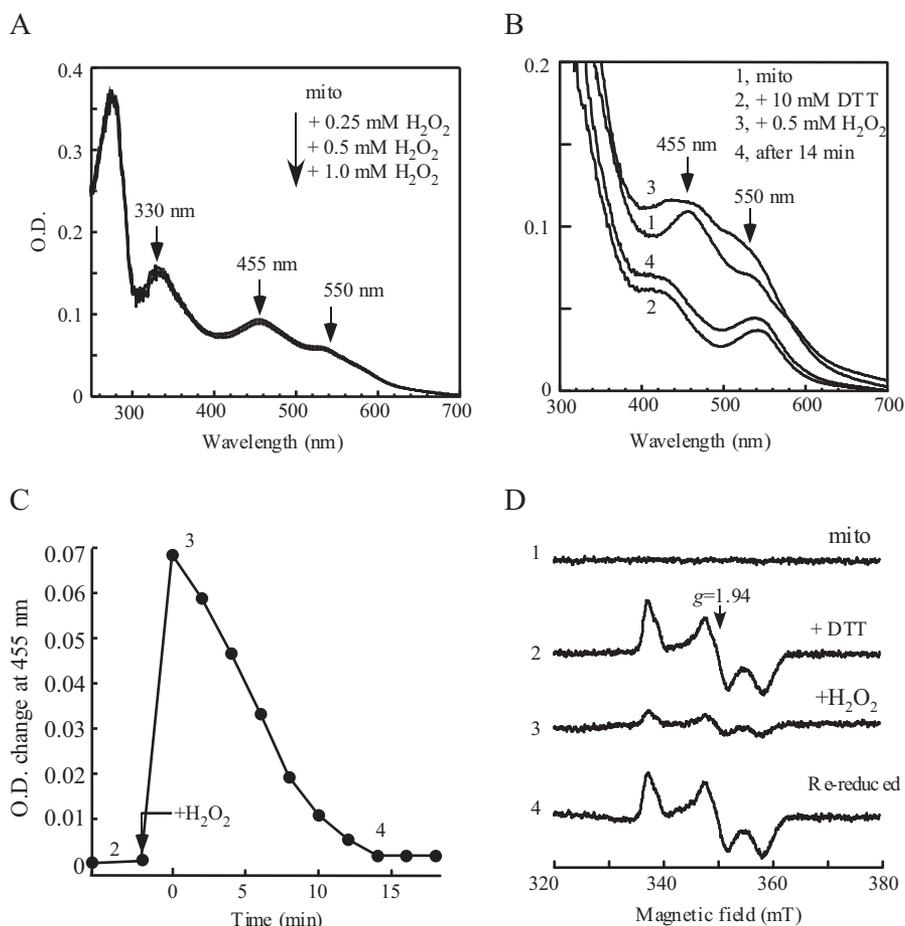


FIGURE 4. **Reversible oxidation of mitoNEET [2Fe-2S] clusters by hydrogen peroxide *in vitro*.** *A*, stability of mitoNEET (mito) [2Fe-2S] clusters in the presence of hydrogen peroxide. UV-visible absorption spectra of mitoNEET [2Fe-2S] clusters were taken after purified mitoNEET [2Fe-2S] clusters (20  $\mu$ M) were incubated with hydrogen peroxide (0 to 1.0 mM). *B*, reversible oxidation of mitoNEET [2Fe-2S] clusters by hydrogen peroxide. UV-visible absorption spectra were taken when purified mitoNEET (20  $\mu$ M) (spectrum 1) was incubated with excess dithiothreitol (10 mM) in an anaerobic cuvette at 37 °C for 20 min (spectrum 2), followed by addition of hydrogen peroxide (0.5 mM) (spectrum 3) and 14 min of additional incubation (spectrum 4). *C*, time course of rereduction of oxidized mitoNEET [2Fe-2S] clusters by dithiothreitol. The absorption peak at 455 nm of the oxidized mitoNEET [2Fe-2S] clusters was plotted as a function of incubation time after addition of hydrogen peroxide. *D*, EPR spectra of mitoNEET [2Fe-2S] clusters. Purified mitoNEET [2Fe-2S] clusters (20  $\mu$ M) (spectrum 1) were reduced with dithiothreitol (10 mM) (spectrum 2), oxidized by hydrogen peroxide (0.5 mM) (spectrum 3), and rereduced after additional incubation with dithiothreitol for 14 min (spectrum 4). mT, millitesla.

membranes because addition of pioglitazone to the cell extracts prepared from *E. coli* cells effectively inhibits the thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters in the cell extracts (data not shown).

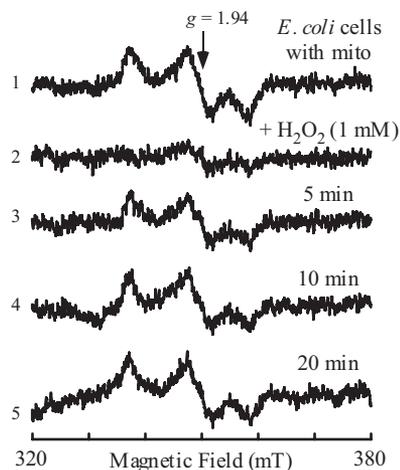
## DISCUSSION

In this study, we report that human mitoNEET [2Fe-2S] clusters are fully reduced when expressed in *E. coli* cells under normal growth conditions and that the mitoNEET [2Fe-2S] clusters can be partially reduced by monothiols and fully reduced by dithiothreitol and the *E. coli* thioredoxin-1 reduced by thioredoxin reductase and NADPH *in vitro*. Importantly, the thiol-reduced mitoNEET [2Fe-2S] clusters can be reversibly oxidized by hydrogen peroxide without disruption of the clusters. Furthermore, binding of the type II diabetes drug pioglitazone effectively blocks the thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters. The results suggest that the mitoNEET [2Fe-2S] cluster may act as a sensor of oxidative signals to regulate mitochondrial function and that the type II diabetes drug pioglitazone may modulate the function of mitoNEET by blocking the thiol-mediated

reduction of the [2Fe-2S] clusters in cells. The possible interplay among biological thiols, hydrogen peroxide, and the type II diabetes drug pioglitazone in regulating the redox state of the mitoNEET [2Fe-2S] clusters is shown in Fig. 7.

The interaction between thioredoxin and iron-sulfur clusters in proteins has been well established in ferredoxin-thioredoxin reductase, where the reduced iron-sulfur clusters provide electrons for reduction of the oxidized thioredoxin (42, 43). However, to the best of our knowledge, the reduction of iron-sulfur clusters in proteins by biological thiols has not been reported previously. The unique ligand arrangement (Cys-72, Cys-74, Cys-83, and His-87) (8–10) and relatively high  $E_{m7}$  value (0 mV) (11, 12) may facilitate the reduction of the mitoNEET [2Fe-2S] clusters by biological thiols because a single mutation (His-87 to Cys) in mitoNEET shifts the  $E_{m7}$  value of the [2Fe-2S] clusters from 0 to  $-320$  mV (11) and effectively prevents the reduction of the clusters by biological thiols (Fig. 3). Unlike NADPH, which fails to reduce the mitoNEET [2Fe-2S] clusters *in vitro* (21), biological thiols have a relatively stable intermediate state of thiol free radical (33, 34) that can provide electrons

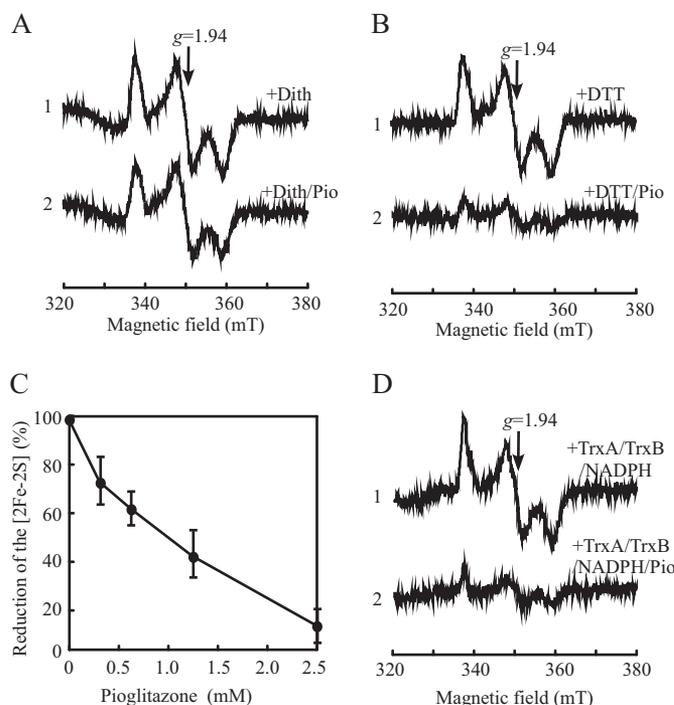
## Reduction of mitoNEET [2Fe-2S] Clusters



**FIGURE 5. Reversible oxidation of mitoNEET [2Fe-2S] clusters by hydrogen peroxide in *E. coli* cells.** *E. coli* cells containing recombinant human mitoNEET (mito) [2Fe-2S] clusters were resuspended in fresh LB medium to an  $A_{600\text{ nm}}$  of 4.0. The cells (spectrum 1) were then treated with hydrogen peroxide (1 mM) (spectrum 2), followed by reincubation in LB medium with aeration at 37 °C for 5 min (spectrum 3), 10 min (spectrum 4), and 20 min (spectrum 5). The EPR signal at  $g = 1.94$  indicates the reduced mitoNEET [2Fe-2S] clusters in *E. coli* cells. The results are representative of three independent experiments. *mT*, millitesla.

for the single-electron reduction of the [2Fe-2S] clusters in mitoNEET. Interestingly, monothiols, such as reduced glutathione, L-cysteine, and *N*-acetyl-L-cysteine, can only partially reduce the mitoNEET [2Fe-2S] clusters, even at high concentrations and with extended incubation time. On the other hand, dithiothreitol (Fig. 2) and *E. coli* thioredoxin prerduced with thioredoxin reductase and NADPH (Fig. 3) can fully reduce the mitoNEET [2Fe-2S] clusters. The redox reactions underlying the reduction of the mitoNEET [2Fe-2S] clusters by biological thiols remain to be further investigated. Nevertheless, the finding that the reduced thioredoxin can reduce the mitoNEET [2Fe-2S] clusters is highly significant. Thioredoxin is highly conserved from bacteria to humans (36). In human cells, there are two major thioredoxins: thioredoxin-1, which is located in the cytosol and nucleus, and thioredoxin 2, which is located in mitochondria (44). The  $E_{m7}$  value of human cytosolic thioredoxin-1 is about  $-230$  mV (pH 7.0) (45), which is close to that of *E. coli* thioredoxin-1 ( $-270$  mV) (37). Therefore, human thioredoxin-1 could be responsible for reducing the mitoNEET [2Fe-2S] clusters. Because human thioredoxin 1 has an essential role in the regulation of complex diseases, including cancer (46), cardiovascular disease, type II diabetes, and aging (47), the proposed role of human thioredoxin-1 in reducing the mitoNEET [2Fe-2S] clusters may provide fresh clues for the links between thioredoxin and various human diseases. However, it should be pointed out that other biological thiols, such as glutathione, L-cysteine, and glutaredoxins (48), may also reduce the mitoNEET [2Fe-2S] clusters in cells.

As a novel target of the type II diabetes drug pioglitazone, mitoNEET has a specific interaction with pioglitazone (1). It has been speculated that pioglitazone may modulate the energy metabolism in mitochondria via direct interaction with mitoNEET (49). Previous studies indicated that binding of pioglitazone stabilizes the [2Fe-2S] clusters in mitoNEET (10) and shifts the  $E_{m7}$  value of the [2Fe-2S] clusters from 0 to  $-100$



**FIGURE 6. Pioglitazone inhibits the thiol-mediated reduction of mitoNEET [2Fe-2S] clusters.** **A**, effect of pioglitazone (*Pio*) on the dithionite-mediated reduction of mitoNEET [2Fe-2S] clusters. Purified mitoNEET [2Fe-2S] clusters (10  $\mu\text{M}$ ) were preincubated with 0 (spectrum 1) or 2.5 mM (spectrum 2) pioglitazone for 15 min at 37 °C, followed by addition of sodium dithionite (*Dith*) (2 mM) and incubation for 15 min under anaerobic conditions. *mT*, millitesla. **B**, purified mitoNEET [2Fe-2S] clusters (10  $\mu\text{M}$ ) were preincubated with 0 (spectrum 1) or 2.5 mM (spectrum 2) pioglitazone for 15 min at 37 °C, followed by addition of dithiothreitol (2 mM) and incubation for 15 min under anaerobic conditions. **C**, titration of pioglitazone. Purified mitoNEET [2Fe-2S] clusters (10  $\mu\text{M}$ ) were preincubated with the indicated concentrations of pioglitazone. The amounts of the reduced mitoNEET [2Fe-2S] clusters were measured from the amplitudes of the EPR signal at  $g = 1.94$  and plotted as a function of pioglitazone concentration after incubation with dithiothreitol (2 mM) under anaerobic conditions. **D**, purified mitoNEET [2Fe-2S] clusters (10  $\mu\text{M}$ ) were preincubated with 0 (spectrum 1) or 2.5 mM (spectrum 2) pioglitazone for 15 min at 37 °C, followed by incubation with *E. coli* thioredoxin (*TrxA/TrxB*) system (10  $\mu\text{M}$  *E. coli* thioredoxin, 10  $\mu\text{M}$  thioredoxin reductase, and 500  $\mu\text{M}$  NADPH) at 37 °C for 15 min under anaerobic conditions.

mV (11). Here, we find that binding of pioglitazone effectively inhibits the thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters, likely because of the decreased  $E_{m7}$  value of the [2Fe-2S] clusters in the protein. Thus, pioglitazone may alter the function of mitoNEET by blocking the thiol-mediated reduction of the [2Fe-2S] clusters in cells. A set of novel ligands have been identified recently that may target mitochondrial mitoNEET (50, 51). It would be of interest to explore the effect of these ligands on the thiol-mediated reduction of the mitoNEET [2Fe-2S] clusters.

The finding that the mitoNEET [2Fe-2S] clusters can be reversibly oxidized by hydrogen peroxide without disruption of the clusters strongly suggests that the mitoNEET [2Fe-2S] cluster may act as a redox sensor to oxidative signals. The redox state of the [2Fe-2S] clusters in mitoNEET has already been attributed to regulating the iron-sulfur cluster transfer from mitoNEET to target proteins (20, 21). It would also be possible that the redox state of the [2Fe-2S] clusters may directly modulate the energy metabolism in mitochondria via specific protein-



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