Heme Deficiency Selectively Interrupts Assembly of Mitochondrial Complex IV in Human Fibroblasts

RELEVANCE TO AGING

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Heme deficiency was studied in young and old normal human fibroblasts (IMR90). Regardless of age, heme deficiency increased the steady-state level of oxidants and lipid peroxidation and sensitized the cells to fluctuations in intracellular Ca2+*. Heme deficiency selectively decreased the activity and protein content of mitochondrial complex IV (cytochrome c oxidase) by 95%, indicating a decrease in successful assembly. Complexes I–III and catalase remained intact under conditions of heme deficiency, whereas ferrochelatase was up-regulated. Complex IV is the only heme protein in the cell that contains heme α, which may account for its susceptibility. The rate of removal and assembly of complex IV declines with age. These findings are relevant to worldwide iron deficiency in women and children and to an age-related decline in complex IV in Alzheimer’s disease patients.

Iron is incorporated into iron-sulfur clusters and into protoporphyrin IX by ferrochelatase to generate heme. Heme and iron-sulfur clusters are the two main iron-containing prosthetic groups that catalyze biochemical reactions. Mitochondria play a major role in assimilation of both forms of iron (1–4). The focus of this study is heme synthesis, which occurs in all cells and is catalyzed by eight enzymatic reactions, four mitochondrial and four cytosolic. The first and the last three reactions of heme synthesis are mitochondrial. The last reaction in the pathway of heme synthesis is the assimilation of iron into protoporphyrin IX to give protoheme (5–7). This reaction is catalyzed by ferrochelatase, an iron-sulfur-containing enzyme, present in the inner membrane of the mitochondria with its active site facing the matrix (8, 9). Thus, first the product (protopheme) is produced in the matrix and then is distributed to different locations in the mitochondria and in the cytosol to be assembled into heme-dependent proteins.

Four types of heme are known in eukaryotes: protoheme and hemes α–c (4). Protopheme is a pool of “free heme” that is not associated or is only loosely associated with proteins (i.e. tryptophan pyrroline) (10) and is the precursor for the other types of heme. Hemes b and c are essentially similar to protoheme, with minor modifications that take place as it is incorporated into each specific protein (4). Catalase, cytochrome P450, hemoglobin, and mitochondrial complexes II and III have heme b, whereas heme c occurs in cytochrome c and cytochrome c1 of complex III. Heme α, on the other hand, is synthesized from protoheme by two modifications: farnesylation and addition of a formyl group to position 8 of the protoheme (11, 12). Two groups of heme α are in cytochrome c oxidase (complex IV), which is an enzyme complex that contains 13 subunits. Hemes α1 and α2 are associated with subunit I of complex IV (4). Complex IV regulates mitochondrial respiration in vivo by cAMP-dependent phosphorylation, dephosphorylation, and nitric oxide (13, 14).

Cytochrome c oxidase is the terminal oxidase in the mitochondrial electron transport chain (ETC).1 The ETC consists of four complexes (I–IV), all in the inner membrane of the mitochondria. Complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) oxidize mitochondrial NADH and succinate, respectively, to form ubiquinol. Ubiquinol is then oxidized to ubiquinone by complex III (ubiquinol:cytochrome c oxidoreductase), which reduces cytochrome c. Cytochrome c then delivers electrons, one at the time, to complex IV to reduce molecular oxygen to water. The four complexes are essential for production of ATP by oxidative phosphorylation (4). The ETC is considered the main source of free radicals (15, 16) that contribute to mitochondrial oxidative damage, which is associated with aging and some diseases (17–21).

Most of what is known about the assembly of complex IV, although not complete, is based on prokaryote and yeast systems (22–24). Assembly of the multisubunits of cytochrome c oxidase with its metal centers (two heme α molecules, two copper centers, and one magnesium atom) (25, 26) proceeds in the mitochondria with a defined sequence of events (27). The rate-limiting step in the assembly of complex IV is assimilation of heme α with subunit I (forming subcomplex S1) and then incorporation of subunits II and IV (forming subcomplex S2) followed by the rest of the subunits until completion of the holocomplex (27). It is not clear if the synthesis of heme α or the process of assimilation into subunit I is rate-limiting in the assembly of complex IV. Unsuccessful folding of the polypeptide (due to lack of a prosthetic group, oxidative damage, or a mutated polypeptide) causes its degradation by proteolysis. Unfolded polypeptides tend to aggregate and compromise the

1 The abbreviations used are: ETC, electron transport chain; NMP, N-methyl protoporphyrin; PDL, population doubling level; DCFH, dichlorodihydrofluorescein; PAGE, polyacrylamide gel electrophoresis; MDA, malondialdehyde.
normal function of the mitochondria (or cell); thus, their protelysis is considered a protective mechanism that prevents unwanted aggregation of unfolded polypeptides (28, 29).

Heme deficiency occurs in porphyria patients and upon aging (30, 31) and in iron deficiency (32), and it is frequent in the elderly under conditions of stress (33). Iron deficiency occurs in an appreciable percentage of the poor women and children in the world. The activity of ferrochelatase declines in the livers of old rats, and little is known about the anabolism of heme in non-hematopoietic tissues, especially upon aging. Complex IV declines with age (34–37) and in Alzheimer's disease patients (38–40). Three of the 13 subunits that make complex IV are coded on mitochondrial DNA and translated on mitochondrial ribosomes. The remaining 10 subunits are expressed from nuclear genes, translated on cytosolic ribosomes, and then imported to the mitochondrial matrix (41).

Since complex IV contains a unique type of heme (heme a), we investigated the effect of heme deficiency on complex IV and other heme-dependent proteins. Heme synthesis decreases in the livers of old rats compared with young rats (30) and in hematopoietic tissues in humans (33). The consequence of abnormal anabolism of heme in non-hematopoietic tissues has been little studied. In this study, we established an in vitro model to evaluate the effect of heme deficiency on different hemoproteins. Heme deficiency was induced by inhibiting ferrochelatase with N-methyl protoporphyrin (NMP) (42). We used normal human lung fibroblasts (IMR90 cells). These cells are used as a model for senescence in vitro. At low population doublings (PDLs), they are considered young (in general, 20–30 PDLs); and at high PDLs, they are old or senescent (in general, >50 PDLs). We found that cytochrome c oxidase (complex IV) was selectively lost by heme deficiency and that its assembly was compromised in old cells compared with young cells. In addition, we found that the rate of proteolysis of unfolded and damaged proteins decreased in mitochondria of old cells compared with young cells.

EXPERIMENTAL PROCEDURES

Materials—NMP and heme were from Porphyrin Products (Logan UT). Mouse anti-bovine cytochrome oxidase subunit IV monoclonal antibody (20E8-C12), mouse anti-human cytochrome oxidase subunit II monoclonal antibody (12c4-F12), mouse anti-human cytochrome oxidase subunit I monoclonal antibody (1D6-E1-A5), mouse anti-bovine NADH:ubiquinone oxidoreductase subunit 2 and ubiquinol cytochrome c oxidoreductase subunit I monoclonal antibody (20c11-B11), mouse anti-bovine ubiquinol:cytochrome c oxidoreductase 39-kDa subunit monoclonal antibody (20c11-B11), mouse anti-bovine ubiquinol:cytochrome c oxidoreductase complex I subunit C monoclonal antibody (13G12-AF12-B11), and dichlorodihydrofluorescein diacetate (DCFH) were from Molecular Probes, Inc. (Eugene, OR). Neat rabbit anti-human ferrochelatase serum (catalog no. AB-FC-1) was from EnVirtue Biotechnologies (Walnut Creek, CA). 5-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrogen peroxide, 2,6-dichloroindophenol, cytochrome c, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (IMR90) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Stock cultures were grown in 100-mm Corning dishes at a PDL of 10.85. The PDLs were calculated as log2(H9262/H11022), where H9262 or H11022—

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Heme deficiency was established by treating young human lung fibroblasts with NMP, an inhibitor of ferrochelatase. Under these conditions (5–10 μM NMP), only 15% loss in viability was observed (p < 0.014) (also see Fig. 3), indicating that NMP is only mildly toxic in vitro. Heme deficiency (10 μM NMP) decreased cytochrome c oxidase (complex IV) by 95% (p < 0.0004) (Fig. 1). Complex II was decreased less (by 30%), and catalase was decreased by 18% (p < 0.01) (Fig. 1). No direct inhibition of cytochrome c oxidase was seen with NMP as evaluated by including NMP in the assay buffer (data not shown). Of the heme proteins assayed, complex IV was affected most strongly by heme deficiency.

Heme Deficiency Is Associated with Oxidative Stress as Evaluated by Oxidation of DCFH and by Lipid Peroxidation—Fluorescence emitted from oxidized DCFH was higher in fibroblasts that were heme-deficient compared with control cells (Fig. 2A). No direct oxidation of DCFH by NMP was seen (data not shown). Thus, the steady-state level of oxidants was high in

RESULTS

Heme Deficiency Induced by NMP Selectively Decreases the Activity of Cytochrome c Oxidase—Heme deficiency was established by treating young human lung fibroblasts with NMP, an inhibitor of ferrochelatase. Under these conditions (5–10 μM NMP), only 15% loss in viability was observed (p < 0.014) (also see Fig. 3), indicating that NMP is only mildly toxic in vitro. Heme deficiency (10 μM NMP) decreased cytochrome c oxidase (complex IV) by 95% (p < 0.0004) (Fig. 1). Complex II was decreased less (by 30%), and catalase was decreased by 18% (p < 0.01) (Fig. 1). No direct inhibition of cytochrome c oxidase was seen with NMP as evaluated by including NMP in the assay buffer (data not shown).
fibroblasts under conditions of heme deficiency. Lipid peroxidation was evaluated by MDA. The steady-state level of MDA was doubled in fibroblasts maintained under conditions of heme deficiency (with NMP) compared with the control fibroblasts \( (p < 0.015) \) (Fig. 2B).

Heme-deficient cells were also sensitive to fluctuation in intracellular Ca\( ^{2+} \) as induced by thapsigargin, which increases cytosolic Ca\( ^{2+} \). When IMR90 cells were treated for 2 h with 200 nM thapsigargin, 50 and 85% killing was observed in heme-sufficient and heme-deficient cells, respectively (Fig. 3). All the previously described experiments were carried out with young to middle-aged cells.

**Assembly of Cytochrome c Oxidase Is Selectively Compromised by Heme Deficiency**

Monoclonal antibodies against proteins of subunits I, II, and IV of cytochrome \( c \) oxidase were used to evaluate assembly in both old and young cells. Under conditions of heme deficiency, a 40 \( \pm \) 14% \( (n = 4) \) decrease in the cellular level of subunit I was observed (Fig. 4A). The levels of subunits II and IV were almost not detectable in heme-deficient cells and are compared with heme-sufficient cells (Fig. 4A).

The levels of cytochrome \( c \) and selected subunits of complexes I and III were also evaluated (Fig. 4B). In two experiments, we found that cytochrome \( c \) decreased 23 and 38% in heme-deficient cells compared with control cells (Fig. 4B). Monoclonal antibodies for subunit ND39 (complex I) and subunit COR I (complex III) were also used. No difference in the levels of these subunits between heme-deficient and heme-sufficient cells was observed (Fig. 4B). The results shown are from young cells.

**Senescence-dependent Decline in the Ability of Human Fibroblasts to Turn Over and Assemble Complex IV**—The selective susceptibility of complex IV to heme deficiency was used to compare old and young cells for the rate of loss of the complex from mitochondria (Fig. 5A). The level of subunit II, as representative for complex IV, was monitored at different intervals after applying NMP to the cells. All of the experiments were done in confluent cells. Extraction of the absorbance of each band shows that the rate of removal of complex IV was 0.503 \( \pm \) 0.03 A units/band/h in old cells compared with 1.48 \( \pm \) 0.37 A units/band/h in young cells (means \( \pm \) S.E., \( n = 4; p < 0.028 \) by the Mann-Whitney non-parametric test) (Fig. 5A). Old cells...
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were about three times slower than young cells in removing complex IV.

Assembly of complex IV was evaluated in old and young cells during restoration of the complex after 1 week of treatment with NMP. NMP was then washed out, and the cells were maintained in fresh medium without NMP. At different intervals after washing out NMP, the level of subunit II was evaluated by Western blotting. In young cells, assembly of complex IV started after 24 h, whereas in old cells, it started after 48 h from washing out NMP (Fig. 5B). Extraction of the absorbance of each of the bands from the different intervals clearly showed a lag of ~24 h between the assembly of complex IV in old cells compared with young cells.

Senescence-dependent Increase in the Level of Mitochondrial Ferrochelatase—Antibody against human ferrochelatase was used to evaluate the level of the enzyme in the mitochondria of old and young cells. Different batches of IMR90 cells were compared. The level of ferrochelatase in young cells was consistently lower than the level in old cells (p < 0.03) (Fig. 6). The level of ferrochelatase was up-regulated in both types of cells treated with NMP (Fig. 6).

**DISCUSSION**

During ATP production by oxidative phosphorylation, electrons from NADH or succinate are transferred through the ETC (complexes I–IV) and reduce molecular oxygen to water (4), a reaction catalyzed by complex IV. The age-related changes in mitochondria are characterized by a decrease in the activity of the ETC, in oxygen consumption, and in cardiolipin and an increase in the generation of free radicals (34, 35, 37, 48–51). A fraction of electrons leak from the ETC and reduce O2 to the O2 radical and H2O2 (15, 20, 52); thus, mitochondria are considered the main endogenous source for the formation of superoxide radical (19, 53).

In this work, we studied the role of heme deficiency in the integrity of the mitochondrial ETC. The influence of heme deficiency on heme proteins was studied in human lung fibroblasts from old and young cells. A known model for the study of heme deficiency uses inhibition of ferrochelatase, which catalyzes the last step of heme synthesis, the incorporation of iron (54–56).

Induction of heme deficiency in IMR90 cells has a profound effect on mitochondria, particularly the assembly of mitochondrial cytochrome c oxidase (complex IV), the heme α-containing complex, which was selectively prevented (Fig. 1). Under conditions of long-term heme deficiency, the activity and level of subunits II and IV of cytochrome c oxidase were almost undetectable compared with those in control cells, regardless of age of the cells (Figs. 1 and 4A). Mitochondrial complexes I–III and catalase were much less affected by heme deficiency, suggesting that heme b- or heme c-dependent proteins and iron-sulfur proteins are not affected by heme deficiency.

Previous studies using NMP as an inhibitor of ferrochelatase show that the enzymatic activity decreases to 15% of the control (55). Thus, inhibition of ferrochelatase leaves the cell with a shortage of protoheme, the precursor for the different types of heme. The type of heme in catalase (which was only slightly affected by heme deficiency) and complex II is heme b, and complex III contains both hemes b and c, whereas complex IV contains heme a. Hemes b and c are essentially similar to protoheme and thus chemically are not modified before assimilation in the corresponding apoprotein. In contrast, heme a is modified by addition of farnesyl and formyl groups to protoheme.
acquires a farnesyl group (a hydrocarbon chain of 15 carbons), and the methyl group at position 8 of protoheme is oxidized to a formyl (aldehyde) group (11, 12). Cytosolic and mitochondrial enzymes catalyze the maturation of protoheme to heme $\alpha$ (57, 58), suggesting that heme $\alpha$ shuttles from the cytosol to the mitochondria to be incorporated into subunit I of cytochrome c oxidase. A plausible explanation for the selective loss of complex IV is that the metabolic complexity of maturation of protoheme to heme $\alpha$, compared with other hemes ($b$ and $c$), is rate-limiting; and under conditions of heme deficiency, less heme $\alpha$ is formed due to the low affinity of the heme $\alpha$ maturation pathway toward protoheme relative to the pathways that assimilate hemes $b$ and $c$ into their apoproteins. Since heme deficiency is also associated with oxidative stress, it is also plausible that oxidative damage inhibits the heme $\alpha$ maturation pathway. Either one of these possibilities would decrease the synthesis of heme $\alpha$, which is consistent with this heme being rate-limiting for the assembly of complex IV.

Part of the decrease (30%) in the activities of complex II and cytochrome c (23–38%) (Fig. 4B) could also result from down-regulating complex II and cytochrome c as a response to alterations in electron flow in the ETC. The decrement in the activity of complex IV decreases electron flow to molecular oxygen and thus induces reduction of ETC complexes (I–III), which are upstream of complex IV. Under these conditions, electrons leak from the ETC to molecular oxygen and generate superoxide radical (15, 52). This is likely to be the mechanism of the elevated oxidants that is seen in heme-deficient cells. Elevated oxidative stress (oxidation of DCFH) and markers of oxidative damage (level of MDA) (Fig. 2, A and B) were found in cells deficient in heme, which supports evidence for oxidative stress in heme-deficient cells.

Subunits II and IV of complex IV did not accumulate in heme-deficient cells (Fig. 4A), probably due to mitochondrial proteolytic enzymes that remove unfolded polypeptides. Two atoms of copper and two molecules of heme $\alpha$ were assimilated in subunits II and I of complex IV, respectively. Copper efficiently reacts with hydrogen peroxide to generate hydroxyl radical that reacts at a diffusion-limited rate with amino acids near the site of its generation, which in this case would be subunit II. This is known as a site-directed Fenton reaction (59). This would contribute to the slow assembly of subunits II and IV, as they depend on each other for assembly. Alteration of amino acids in the unfolded polypeptide would induce their degradation and removal by mitochondrial proteases. It is known that unfolded or damaged mitochondrial polypeptides are subject to elimination by specific proteases (28, 29, 60).

Heme-deficient cells maintain relatively high levels of subunit I of complex IV (Fig. 4A), suggesting that the cells keep producing this subunit in an attempt to assemble the holocomplex. It is likely that this level of subunit I reflects a balance between its production and degradation due to lack of successful assembly. This is consistent with the functions assigned to subunit I and heme $\alpha$ as the initiators of the assembly of complex IV.

Heme deficiency has been used as a model to study the effect of senescence on quality control and maintenance of mitochondria, a topic that is very relevant to aging (53). With long-term heme deficiency (7 days), the levels of subunits II and IV of cytochrome c oxidase were undetectable. We followed the level of complex IV during short-term heme deficiency (24 h) and found a senescence-dependent decrease in the rate of elimination of complex IV (Fig. 5A). This suggests a senescence-dependent decrease in the ability of mitochondria to turn over unfolded subunits II and IV and probably damaged proteins in general that could result in their accumulation (61). Simultaneously, we followed the recovery of complex IV after long-term heme deficiency by removing the inhibition (Fig. 5B). We found a senescence-dependent lag of 24 h in the assembly of complex IV. The data are from one of at least four independent experiments using different batches of cells.
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IV, suggesting a decline in the ability of mitochondria from old cells to establish a new complex of cytochrome c oxidase. As assembly of complex IV (and other ETC complexes) relies on proteins that are imported from the cytosol into the mitochondria, Protein import into mitochondria is sensitive to oxidative stress (62), which is elevated under conditions of heme deficiency. Senescent cells have a reduced ability to cope with and recover from oxidative stress (63–66). Thus, the slow rate of elimination of proteins and the slow rate of assembly of ETC complexes in old cells compared with young cells suggest a senescence-dependent decline in mechanisms of quality control and maintenance of mitochondria.

Oxidants and damage to the ETC could alter proper regulation of Ca\(^{2+}\) by the mitochondria (67). Heme-deficient cells were susceptible to fluctuations in intracellular Ca\(^{2+}\), as estimated by their sensitivity to thapsigargin, which inhibited Ca\(^{2+}\) uptake by the endoplasmic reticulum, causing elevation of cytosolic Ca\(^{2+}\) (Fig. 3). This observation could have implications for Ca\(^{2+}\)-dependent toxicity of neuronal cells, which continuously metabolize Ca\(^{2+}\). NMP also up-regulated ferrochelatase, which is likely to be an attempt to overcome the lack of iron for ferrochelatase to incorporate into protoporphyrin IX. Iron deficiency has been reported to decrease the activity of cytochrome c oxidase as well as other iron-related enzymes (32, 33). It is likely that iron and heme deficiencies overlap in their influence on mitochondria, especially in their effect on complex IV. Research on heme metabolism, regulation, and deficiency in non-hematopoietic tissues should contribute to the understanding of the mitochondrial decay in aging, particularly of the brain.

Acknowledgment—We are grateful to E. Roitman for technical help.

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36. It is not clear why complex IV declines with age and in Alzheimer’s disease. Complex IV is the only hemoprotein that contains heme a, and it could be that this type of heme declines with age. To our knowledge, heme a has not been studied as a function of age in the brain. Levels of heme a in Alzheimer’s disease patients decreased by 22% (although this was not significant when measured as the difference in oxidized and reduced spectra of heme), whereas hemes b and c were not changed (decreased by 4 and 7%, respectively) compared with healthy age-matched controls (71). Heme a forms a small fraction of the total heme in the cell; thus, we believe that the lack of a sensitive and analytical approach to measure heme a leaves the question about levels of heme a in tissues during aging unanswered.

Iron deficiency induces heme deficiency and anemia due to the lack of iron for ferrochelatase to incorporate into protoporphyrin IX. Iron deficiency is present in 7–2 billion women and children and causes oxidative damage and loss of function in mitochondria and mitochondrial DNA (72). Iron deficiency has been reported to decrease the activity of cytochrome c oxidase as well as other iron-related enzymes (32, 33). It is likely that iron and heme deficiencies overlap in their influence on mitochondria, especially in their effect on complex IV. Research on heme metabolism, regulation, and deficiency in non-hematopoietic tissues should contribute to the understanding of the mitochondrial decay in aging, particularly of the brain.

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