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Review

# Heme, iron, and the mitochondrial decay of ageing

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**Abstract**

Heme, the major functional form of iron, is synthesized in the mitochondria. Although disturbed heme metabolism causes mitochondrial decay, oxidative stress, and iron accumulation, all of which are hallmarks of ageing, heme has been little studied in nutritional deficiency, in ageing, or age-related disorders such as Alzheimer's disease (AD). Biosynthesis of heme requires Vitamin $B_6$, riboflavin, biotin, pantothenic acid, and lipoic acid and the minerals zinc, iron, and copper, micronutrients are essential for the production of succinyl-CoA, the precursor for porphyrins, by the TCA (Krebs) cycle. Only a small fraction of the porphyrins synthesized from succinyl-CoA are converted to heme, the rest are excreted out of the body together with the degradation products of heme (e.g. bilirubin). Therefore, the heme biosynthetic pathway causes a net loss of succinyl-CoA from the TCA cycle. The mitochondrial pool of succinyl-CoA may limit heme biosynthesis in deficiencies for micronutrients (e.g. iron or biotin deficiency). Ageing and AD are also associated with hypometabolism, increase in heme oxygenase-1, loss of complex IV, and iron accumulation. Heme is a common denominator for all these changes, suggesting that heme metabolism maybe altered in age-related disorders. Heme can also be a prooxidant: it converts less reactive oxidants to highly reactive free radicals. Free heme has high affinity for different cell structures (protein, membranes, and DNA), triggering site-directed oxidative damage. This review discusses heme metabolism as related to metabolic changes seen in ageing and age-related disorders and highlights the possible role in iron deficiency.


*Keywords:* Iron; Neurodegeneration; TCA cycle; Complex IV; Succinyl-CoA; Ferrochelatase

*Abbreviations:* TCA, tricarboxylic acid (Krebs) cycle; AD, Alzheimer's disease; HO, heme oxygenase; ALA, δ-aminolevulinic acid; ALAS, δ-aminolevulinic acid synthetase; HRM, heme regulatory motif; PPIX, protoporphyrin IX; FC, ferrochelatase; SCS, succinyl-CoA synthetase; Complex IV, cytochrome *c* oxidase; CNS, central nervous system; ETC, electron transport chain; APP, amyloid precursor protein

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## 1. Introduction

The two major functional forms of iron in eukaryotic cells are heme (ferriprotoporphyrin IX) and iron–sulfur clusters (Atamna et al., 2002a); iron is incorporated into both in the mitochondria (Ponka, 1999; Lill and Kispal, 2000).

Biosynthesis of the tetrapyrrole ring of heme (protoporphyrin IX) starts in the mitochondrial matrix by condensing succinyl-CoA (donated from the TCA cycle) with glycine (from the mitochondrial pool of the amino acid) to form δ-aminolevulinic acid (ALA). ALA is then exported to the cytoplasm where δ-aminolevulinic acid dehydratase condenses 2 ALAs to porphobilinogen. Four molecules of porphobilinogen are then converted to protoporphyrin IX through decarboxylation and oxidation steps, which begin in the cytosol and returns to the mitochondria. Ferrochelatase (FC), an iron–sulfur enzyme in the inner membrane of the mitochondria (Dailey et al., 2000), catalyzes the last step in heme biosynthesis by inserting iron into protoporphyrin-IX (PPIX) to produce heme in the mitochondria (Ponka, 1999). The biosynthesis of one mole of tetrapyrrole ring requires 8 moles of succinyl-CoA and 8 mole of glycine.

Heme plays catalytic and regulatory roles in all cells (Zhu et al., 2002). It controls the activity of specific transcription factors (Snyder et al., 1998; Ogawa et al., 2001) and other proteins in a variety of biochemical pathways (McCoubrey et al., 1997; Goessling et al., 1998; Tang et al., 2003) by binding to the "heme regulatory motifs" (HRM) (Zhang and Guarente, 1995). Thus, fluctuations in endogenous heme may also be important for the regulation of cellular metabolism (Atamna et al., 2002b; Zhu et al., 2002). The regulatory role of heme in erythroid cells has been known for a long time (Zhu et al., 1999).

Several conditions can alter heme biosynthesis and therefore decrease or increase cellular content of heme. Deficiency for specific vitamins (e.g. Vitamin $B_6$, riboflavin), minerals (e.g. iron, zinc), exposure to high dose of specific toxins (e.g. aluminum, lead) (Daniell et al., 1997), anoxia (Vijayasarathy et al., 2003), or fever (Kihara et al., 1999) can decrease heme biosynthesis. Heme content, on the other hand, increases in response to estrogen (Brawer et al., 1978), as iron availability increases (Laskey et al., 1986), or after long-term exposure to a very low level of lead (blood level 0.7 μg/dl) (Iavicoli et al., 2003). Increased demand for the production of heme occurs also during exposure to toxins that require detoxification by cytochrome P450 system, the intrauterine development of the fetus (Woods, 1976), and probably ageing (see further).

Decreased heme biosynthesis in erythroid cells causes anemia. The consequences of decreased heme levels in non-erythroid cells are loss of mitochondrial complex IV, oxidative stress, accumulation of iron, and cell death whenever stimulation by growth factors occurs (Amuro et al., 1984; Atamna et al., 2001, 2002b; Killilea et al., 2003). The consequences of changes in heme in vivo, especially on the brain are not fully known. The activity of complex IV is tightly coupled to neuronal metabolic activity (Nakao et al., 1998), therefore brain may be the most susceptible organ for a decrease in complex IV and for oxidative stress, which occur in ageing and in Alzheimer's disease. The consequences of increased tissue content of heme, on the other hand, are not known. High levels of heme induce heme oxygenase-1 and if heme persists it may also contribute to oxidative damage.

The capacity of heme biosynthesis and regulation varies among different tissues, although micronutrient requirements and inhibitors are identical. Erythroid precursors have

the highest rate of heme production among all nucleated cells, followed by hepatocytes. Heme biosynthesis in the brain, on the other hand, varies according to the type of brain cell. Interestingly, heme biosynthesis appears to be higher in non-neuronal cells and lower in neuronal cells (Whetsell et al., 1978). Disturbed heme metabolism can lead to iron accumulation (Nakajima et al., 1999; Atamna et al., 2002b). For example, alterations in heme metabolism, in conjunction with the disturbed iron metabolism, may occur in Alzheimer disease (Smith et al., 2000). The nature of these changes is not known. This review implicates changes to biosynthesis of the organic part of heme (e.g. tetrapyrrole ring), together with iron in mitochondrial decay, oxidative stress, and hypometabolism seen in ageing and in Alzheimer's disease.

## 2. Net loss of succinyl-CoA due to porphyrin biosynthesis: possible contribution to hypometabolism of ageing

Heme is tightly connected to the TCA cycle and to a large number of metabolic processes of the cell. Glycolysis and oxidative phosphorylation decrease in the brain with ageing as well as in AD patients (Blass, 2001; Valla et al., 2001). The consequences of these metabolic changes on the TCA cycle (e.g. level of succinyl-CoA) and heme as well as the factors that trigger hypometabolism are not clear.

The TCA cycle is an amphibolic pathway; the intermediates of the TCA cycle are involved in anabolic and catabolic processes by supplying or receiving the carbon skeleton of several metabolites (Owen et al., 2002) (Fig. 1). The intermediates, oxaloacetate, α-ketoglutarate, citrate, and succinyl-CoA, of the TCA cycle are the precursors for several anabolic processes, including the biosynthesis of porphyrins, amino acids, purines, pyrimidines, fatty acids, sterols, and some neurotransmitters. These metabolites, except for the porphyrins, provide the TCA cycle with the intermediates when turned over by the metabolic activity of the cell (Fig. 1). Some of these metabolites such as the amino acids and fatty acids are consumed in the diet, while porphyrins and heme are not bioavailable from the diet and must be synthesized in situ. Therefore, in most of these metabolic activities, the TCA cycle reclaims the complete or partial intermediates by salvage or through a recycling mechanism.

### *2.1. Porphyrin biosynthesis causes net loss of TCA intermediates*

No biological function has been ascribed for the porphyrins, other than as a precursor for heme (Fig. 1). Unlike the recycled TCA intermediates, porphyrins are continuously excreted from the body. Porphyrins usually leave the body in two ways: (1) they are continuously excreted in the urine and (2) when heme is turned over to bilirubin, which is excreted as bile pigment. Therefore a net efflux of succinyl-CoA from TCA cycle occurs as porphyrins are synthesized (Fig. 1).

The biosynthesis of one mole of heme requires 8 mole of succinyl-CoA (Woods, 1976; Ponka, 1999) from the TCA cycle and 8 mole of glycine from the mitochondrial pool of amino acids. As not all the porphyrin produced by the heme biosynthetic pathway proceed to become heme (some porphyrin side products are excreted), in reality, the quantity of succinyl-CoA that leaves the TCA cycle for porphyrin biosynthesis exceeds 8 mole/1 mole

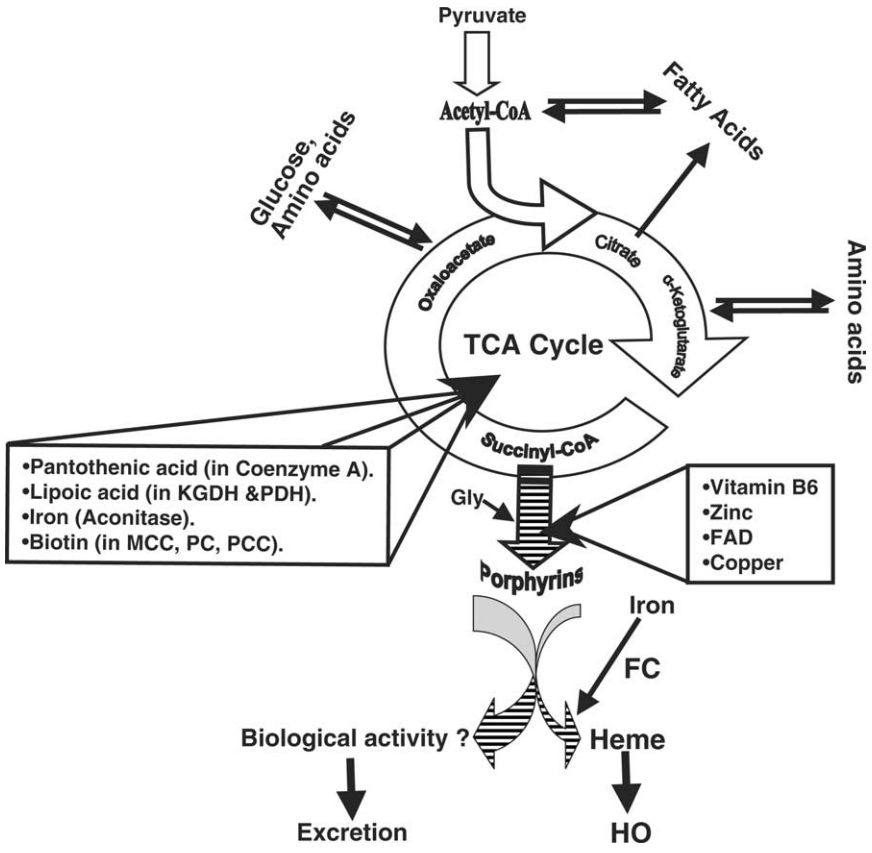


Fig. 1. The major metabolic pathways of the TCA cycle, biosynthesis of porphyrins, and the essential micronutrients. Vitamins and micronutrients are grouped in two boxes connected to arrowheads, which direct to the respective metabolic pathway. Striped arrows indicate the porphyrin biosynthesis starting from succinyl-CoA, heme biosynthesis, and their excretion. Black arrows indicate the direction of metabolites flow from or to the TCA intermediates. FC; ferrochelatase, HO; heme oxygenase, PC; pyruvate carboxylase, PCC; propionyl-CoA carboxylase, MCC; 3-methyl crotonyl-CoA carboxylase, CoA; coenzyme A, KGDH; α-ketogluterate dehydrogenase, PDH; pyruvate dehydrogenase, Gly; Glycine. The question mark (?) suggests a possible biological relevance for the porphyrin side products.

heme. It is well known that the porphyrin synthetic pathway produces a mixture of porphyrin side products (e.g. uroporphyrin I & III, coproporhyrin I & III) (Ponka, 1999). In rodents (F-344), about 2 nmol/day (Bowers et al., 1992) of total porphyrins are excreted in urine; this figure increases 100–1000 fold in humans (Daniell et al., 1997). Only 5% of these porphyrins are converted to heme, as demonstrated in rat primary olfactory receptor neurons (Ingi et al., 1996). In addition, it is estimated that between 20 and 35% of newly formed heme is directly converted to bile pigments (Grandchamp et al., 1981), suggesting a continuous demand for heme biosynthesis. Therefore, it appears that the synthetic pathway of porphyrins is energy consuming and drains much succinyl-CoA from the TCA cycle.

The steady-state concentration of succinyl-CoA in isolated mitochondria is estimated at between 0.2 and 1.6 nmols/mg mitochondrial proteins (Quant et al., 1989) or 0.04–0.32 nmol/mg total protein, as mitochondrial protein is 1/5 of the tissue's protein (Tyler, 1992). The heme content in rat tissues has been estimated to range between 1 and 1.5 nmol/mg protein (Ingi et al., 1996). This range appears to be an overestimation caused by not considering the heme from hemoglobin, which contaminates the tissues during preparations.

We have measured heme in human brains free of hemoglobin using HPLC and found it $0.19 \pm 0.08$ nmol/mg total protein ($n = 6$ (Atamna et al. in preparation; Parker et al., 1994). Therefore, the amount of succinyl-CoA that should be consumed from the TCA cycle needed to account for the 0.19 nmol/mg heme is 1.52 nmols in brain, given that each mole of heme requires 8 mole of succinyl-CoA. This estimation is the minimal amount, as we did not account for the excreted porphyrin side products, which likely to vary between the different types of tissues. Although the level of succinyl-CoA may vary with the metabolic state of the mitochondria and the source for acetyl-CoA (Hansford and Johnson, 1975), it appears that the level of succinyl-CoA that is available for ALAS may limit heme biosynthesis.

The TCA cycle in the mitochondria may effectively adjust and overcome the possible limitation in the supply of succinyl-CoA by accelerating its production and decreasing its consumption for ATP biosynthesis. To do so, the TCA cycle needs the precursors for acetyl-CoA, which comes from fat and glucose, which are abundant (Fig. 1). More important is an adequate level of iron and the vitamins biotin, lipoic acid, and pantothenic acid, which are necessary for optimal function of the TCA cycle, and therefore the biosynthesis of the porphyrins (Fig. 1). Acute or marginal deficiencies for these vitamins may limit the efficiency of the TCA cycle, even though glucose might be abundant. It is likely that deficiency of any one of the micronutrients that are important for heme biosynthesis would decrease succinyl-CoA.

Given that succinyl-CoA is also an essential metabolite for ATP production, these findings are not expected since they suggest that the production of succinyl-CoA may be hardly enough to keep up with the demand for porphyrins and heme biosynthesis. Since ATP is an essential metabolite for cell survival, we believe that in case of succinyl-CoA limitation ATP production will be prioritized over heme. We have demonstrated that low levels of heme do not immediately decrease cell viability but rather disrupt the cell response to stress or inhibit completion of differentiation (Atamna et al., 2002b). In order to understand the dynamics of the TCA cycle, the pool for porphyrin, and heme—we need to evaluate simultaneously within the same tissue the concentration of succinyl-CoA, the size of the pool of porphyrins, ATP level, and the half-life of heme.

### *2.2. Succinyl-CoA synthetase and heme biosynthesis*

Succinyl-CoA is formed exclusively within the mitochondria by succinyl-CoA synthetase (SCS) coupled with GDP or ADP phosphorylation (Jenkins and Weitzman, 1988). The enzyme also catalyzes the biosynthesis of succinyl-CoA from succinate, which may be useful to elevate content of ALA in the cells to increase heme content (Jenkins and Weitzman, 1988) where extra heme is needed. Jenkins et al. have demonstrated the existence of two isoforms of SCS, the GTP-specific (G-SCS) and ATP-specific isoforms (ASCS) (Jenkins and Weitzman, 1988); confirmed by Johnson et al., (1998). The two isoforms of SCS share

identical alpha subunit and differ in their β subunits, indicating that the β subunit determines the specificity for nucleotide binding.

Jenkins et al. found that G-SCS may be more important for catalysis of the reverse reaction to support heme biosynthesis in diabetes or after inhibition of heme biosynthesis (Jenkins and Weitzman, 1988). Interestingly, the erythroid specific ALAS binds to A-SCS but not to G-SCS, supporting the possible role of A-SCS in heme biosynthesis in erythroid cells (Furuyama and Sassa, 2000), although the effect on heme biosynthesis still needs to be directly demonstrated to confirm this idea. A hypothesis of differential role of SCS isoforms in succinate metabolism is intriguing, and direct assessment of this possibility is needed in the different tissues especially in conditions of increased need for heme biosynthesis.

## 3. Relationship between aconitase, ferrochelatase, succinyl-CoA, and ageing

The level of ferrochelatase (FC) and its activity changes according to the intracellular levels of iron (Taketani et al., 2000), nitric oxide, and probably other free radical species (Furukawa et al., 1995; Sellers et al., 1996). The iron–sulfur cluster in FC appears responsible. FC utilizes only ferrous iron ($Fe^{2+}$) as a substrate, which must reach the active site of FC from the cytoplasmic side of the inner membrane (Lange et al., 1999). Similarly, aconitase, an enzyme in the TCA cycle, responds to levels of iron and free radicals through its iron–sulfur cluster. The cellular location of aconitase in the matrix of the mitochondria suggests it interacts with mitochondrial iron. Additional inner membrane iron–sulfur dependent proteins (e.g. Rieske protein in complex III) were not reported to exchange the iron in their iron–sulfur cluster with free iron. Disruption of the iron–sulfur cluster in aconitase and FC by any of the above mechanisms (iron deficiency or oxidative stress) decreases their activity leading to decrease in heme biosynthesis. Loss of the iron–sulfur cluster in aconitase decreases succinyl-CoA (the precursor for heme), while loss of the iron–sulfur cluster in FC directly inhibits heme biosynthesis (Taketani et al., 2000).

Iron accumulates in senescent cells (Killilea et al., 2003) as it does in vivo and in neurodegeneration (Thompson et al., 2001), while the level of FC increases in senescent cells (Atamna et al., 2001). These two observation may be related as suggested by Taketani et al. (Taketani et al., 2000), where the level of FC correlates with intracellular level of iron. On the other hand, the production of free radicals increases with age, while the level of aconitase, decreases, probably as a result of oxidative stress (Yan et al., 1997). Thus, in senescent cells it seems that oxidative stress decreases aconitase, but not FC, while the increased level of iron appears to increase FC but not aconitase. The reverse direction of change in FC and aconitase with age may cause depletion of the pool of succinyl-CoA from the mitochondria, a situation that could dramatically limit TCA activity. Therefore iron accumulation with age could be a compensatory mechanism to keep adequate levels of heme production (Atamna et al., 2002b, see Section 4). Since succinyl-CoA is also committed to metabolic pathways other than heme biosynthesis (e.g. production of ATP), a decline in its level is presumed to cause a metabolic crisis.

The inhibition of aconitase and FC when iron is low could be a safety mechanism to prevent PPIX and ALA from accumulating in the mitochondria since both metabolites are toxic at high concentrations. High level of iron on the other hand, which synchronizes with

high level of FC, may be a mechanism to accelerate iron excretion by synthesizing heme, which is then degraded by HO in the endoplasmic reticulum (ER). The released iron from heme by HO is believed to be excreted out of the cell (Ponka, 1999; Atamna et al., 2002a). Presumably it would be plausible and safer for the cell to mobilize heme, rather than iron (e.g. ADP-Fe), since several heme-binding proteins have been described that may serve as heme carriers (Ponka, 1999). These proteins could recruit heme to the ER and minimize its prooxidant activity. Synthesizing heme by FC and shuttling it to HO should decrease the size of the labile pool of iron. FC uses the labile pool of iron to generate heme. HO degrades heme in the ER releasing iron, which is then may be excreted from the cell and the antioxidant billirubin.

## 4. Heme and neurodegeneration of Alzheimer's disease

We have noticed that the cytological changes resulting from heme and heme-*a* deficiency induced in human cells in vitro, and in primary hippocampal neurons from rat, mimics part of the cytopathology of the brain during ageing (Atamna et al., 2002b). Therefore we hypothesized that abnormal metabolism of heme (or heme-*a*) may contribute to the mitochondrial decay, oxidative stress, and neurodegeneration of ageing.

Heme metabolism appears altered in ageing and Alzheimer's disease. Heme degradation increases with age, in neurodegeneration, and after exposure to stress (Bitar and Shapiro, 1987; Schipper, 2000; Smith et al., 2000), which could be due, in part, to a high tissue content of heme. The level of bilirubin (one of the products of heme degradation by HO-1) is increased in the CSF in AD patients (Kimpara et al., 2000). Venters et al. (Fawcett et al., 2002) have isolated from AD brains a heme/heme-metabolite, which inhibits the muscarinic acetylcholine receptor by producing oxidants. The level of this inhibitor was threefold higher in AD brains than in controls. Three aspects of heme's function previously mentioned are relevant to Alzheimer's disease. Although heme is important for metabolic homeostasis, mitochondrial integrity, and regulation of specific transcription factors, the role of endogenous heme in cell metabolism has been little studied especially in pathological conditions. A few studies conducted on bone marrow and on liver from rats showed a decrease in ALAS activity and an increase in HO (Bitar and Shapiro, 1987; Kihara et al., 1999). There is also a lack of data on tissue content of heme. Several studies demonstrated that a decrease in heme might lead to metabolic crisis and mitochondrial decay (Kim et al., 2001; Ogawa et al., 2001; Atamna et al., 2002b; Zhu et al., 2002) while little is known on the consequences of increase in heme content on the cell.

### *4.1. Heme and paired helical filaments*

The paired helical filaments (PHF) are hyperphosphorylated proteinaceous aggregates found in the dying cells from AD patients. A PHF-dependent kinase activity has been described within the purified PHF (Vincent and Davies, 1992). Heme has been shown to inhibit PHF phosphorylation catalyzed by PHF-dependent kinase. Heme also abolishes the immunoreactivity of phosphorylated PHF with Alz50, a specific antibody that recognizes phosphorylated tau in PHF (Vincent and Davies, 1992). Hyperphosphorylation of Tau

increases in AD patients. The concentration of heme required to efficiently inhibit PHF phosphorylation is 10 μM. The inhibitory effect of heme on the phosphoryltion of PHF may be promoted by oxidative damage to the kinase in PHF due to the prooxidative nature of heme or might be HRM-dependent in the amino acid sequence of the kinase in PHF. Cyclin-dependent kinase 5 (Cdk 5) is a tau kinase whose activity appears essential for tau hyperphosphorylation (Noble et al., 2003). Cdk 5 may interact directly with heme as it contains a potential HRM in its carboxyl terminal in addition to several histidines, which may bind heme.

### *4.2. Heme and amyloid-β aggregates*

Senile plaques are extracellular aggregates of mainly amyloid-β peptides, observed at high frequency in AD brains. Unidentified conditions in the AD brain cause an accumulation and aggregation of amyloid-β peptides to senile plaques. It appears that some factors may accelerate the aggregation of amyloid-β peptides in vivo. Physiologically relevant metals, such as iron, zinc, or copper, can accelerate amyloid-β aggregation in vitro (Bush, 2003).

Interestingly, heme and other related porphyrins were demonstrated to inhibit in vitro aggregation of amyloid-β even at amyloid-β/heme ratios as low as 1/10 (Howlett et al., 1997). Heme also protected IMR32 (neuronal cells) against the cellular toxicity of amyloid-β (Howlett et al., 1997). Amyloid-β possesses three histidines that can bind with heme, which subsequently may prevent its aggregation either by changing conformation of the peptide, its hydrophobicity, or by masking sites that binds free iron or copper, which may trigger amyloid-β aggregation (Atamna et al., submitted). The effect of endogenous heme on aggregation of amyloid-β is not known.

### *4.3. Heme oxygenase and amyloid precursor protein (APP)*

Heme oxygenases (HO) are ubiquitous and inducible enzymes that degrade heme (Maines, 1988). Heme oxygenase activity increases in AD patients (Smith et al., 2000) and during ageing (Abraham et al., 1985; Bitar and Shapiro, 1987). Elevated HO activity may cause heme depletion (Toda et al., 1996) if heme biosynthesis does not increase. Little research has been conducted on the relationship between heme biosynthesis and degradation. One study showed that induction of HO is not necessarily associated with an increase in heme biosynthesis in old rats (Abraham et al., 1985). Of major interest is that HO is inhibited by APP (both proteins are located in ER) (Takahashi et al., 2000). Inhibition of HO by APP suggests lower production of bilirubin. Interestingly, the level of bilirubin slightly increases in the CSF in AD patients (Kimpara et al., 2000), suggesting that HO inhibition (if it occurs in vivo) has little effect on bilirubin production or heme synthesis increased. We have demonstrated that low levels of intracellular heme causes aggregation of APP (Atamna et al., 2002b). These finding suggest that heme metabolism is connected to APP and that heme oxygenase may serve as a chaperone for APP processing.

### *4.4. Heme inhibits muscarinic acetylcholine receptor (mAchR)*

Fawcett et al. have isolated an irreversible inhibitor for the human muscarinic acetylcholine receptor from the brains of Alzheimer patients (Fawcett et al., 2002). They have

suggested that the inhibitor is heme-related; increases three-fold in AD patients and activates the mAchR by free radical mediated process. Furthermore, HasA a heme-dependent protein has been described in senile plaques from AD patients (Castellani et al., 2000). This protein is a homologue of the bacterial heme transporter, possibly serving as heme carrier protein in human tissue.

### *4.5. Heme and heme-a content in brain of Alzheimer's disease patients*

A pioneering study, Parker et al., (1994) measured heme and heme-*a* in mitochondria isolated from whole brain from AD patients and controls and found no difference in heme content. While mean heme-*a* contents decreased by 22%, this was not statistically significant. We believe a region-specific determination of heme rather than whole brain and compartmentalization of heme and heme-*a* between mitochondria and the cytosol (using new analytical methods) is needed to provide a comprehensive understanding for the role of heme in disturbed iron metabolism and reductions in complex IV in AD.

## 5. Maturation of heme to heme-*a*: relevance to age-dependent complex IV reduction

Mitochondrial complex IV is the only protein in the cell that contains heme-*a* (Mogi et al., 1994) at a stochiometry of two molecules of heme-*a* for each complex IV (Mogi et al., 1994). Heme is converted to heme-*a* through two chemical modifications. The first modification is farnesylation of the pyrrole ring A of heme (Mogi et al., 1994; Barros et al., 2001). The second modification is the oxidation of the methyl group on the pyrrole ring D to a formyl group (Brown et al., 2002). The farnesyl moiety produced from the TCA intermediates is also the precursor for cholesterol, dolichol, and coenzyme Q (Goldstein and Brown, 1990), in addition to farnesylated proteins. This suggests that the maturation of heme-*a* is in competition with different metabolic pathways. Mutations in genes that are essential for heme-*a* maturation decrease the activity of human complex IV (Antonicka et al., 2003), which is consistent with the rate limiting role of heme-*a* in the assembly of the holo-complex IV (Wielburski and Nelson, 1984; Nijtmans et al., 1998). Thus the amount of heme-*a* in the cell determines the rate of the assembly of complex IV.

Three experimental conditions known to limit the production of heme-*a* are (1) when heme production is low (Atamna et al., 2001), (2) a pantothenate deficiency exists (Plesofsky-Vig, 1996), (3) hypoxia (Vijayasarathy et al., 2003). The activity of mitochondrial complex IV decreases (between 30 and 50%) both with ageing and in Alzheimer patients (Parker et al., 1994; Maurer et al., 2000). Changes in metabolism of heme and heme-*a* may lead to loss of complex IV and iron accumulation as has been demonstrated in the model for heme deficiency (Atamna et al., 2002a), suggesting that heme deficiency leads to heme-*a* deficiency.

Heme-*a* has been little studied in relation to ageing or age-related disorders. Maybe measuring total heme in the cell is useful to assess the metabolic status of heme and complex IV. We suggest, however, that the ratio of heme-*a* to total heme could be a useful parameter for evaluation of heme metabolism, especially when there is a necessity for assessment of complex IV. Measuring the ratio heme-*a*/heme in human white blood cells or biopsies may

prove useful as a clinical tool to monitor changes to complex IV and heme metabolism in the body. This could be significant given that heme biosynthesis responds to several chemical and physiological stresses.

## 6. Dependence of heme biosynthesis on micronutrients

Biosynthesis of the tetrapyrrole ring requires several vitamins and minerals (Ponka, 1999). Specific enzymes of the heme biosynthetic pathway directly depend on adequate tissue levels of Vitamin $B_6$, zinc, and flavin. Additionally, heme biosynthesis depends on micronutrients important for producing succinyl-CoA from the Krebs (TCA) cycle, including: biotin, lipoic acid, and pantothenic acid.

Iron and copper are unique in their role in heme biosynthesis. Iron is inserted into protoporphyrin IX by ferrochelatase (FC) to form heme and is also essential for heme biosynthesis in a different way. An iron–sulfur cluster in ferrochelatase (Dailey et al., 2000), which doesn't play a catalytical role in FC, is still essential for its activity. Furthermore iron is in an iron–sulfur cluster of aconitase. Copper, on the other hand, stimulates the activity of ferrochelatase probably by decreasing the Km for iron (Wagner and Tephly, 1975). Therefore copper deficiency alters heme (Williams et al., 1985).

Inadequate intake of these micronutrients due to ageing can disturb heme metabolism and contribute in part to the age-dependent hypometabolism and increased oxidative damage seen with age. Marginal vitamin and mineral deficiencies are common in the population (Wakimoto and Block, 2001; Ames, 2003) with iron being the most common micronutrient deficiency in women and children. Iron deficiency, as well as low levels of other vitamins and minerals (Table 1) may adversely affect heme biosynthesis and mitochondrial metabolism.

Table 1
Essential micronutrients in heme synthesis

| Nutrient | Enzyme | Product | Location | Select refernces |
|---|---|---|---|---|
| Vitamin $B_6$ | δ-Aminolevulinic acid synthetase | δ-Aminolevulinic acid | Mitochondria | Woods (1974) |
| Zinc | δ-Aminolevulinate dehydratase | Porphobilinogen | Cytosol | Pande and Flora (2002) |
| Riboflavin | Protoporphyrinogen oxidase | Protoporphyrin | Mitochondria | Dailey and Dailey (1998) |
| Iron[a] | Ferrochelatase | Protoheme (heme) | Mitochondria | See text |
| Copper | Ferrochelatase | – | Mitochondria | Williams et al. (1985) |
| Biotin | Mitochondrial carboxylases | TCA intermediates | Mitochondria | Mock (1996) |
| Lipoic acid | α-Ketoglutarate dehydrogenase and pyruvate dehydrogenase | TCA intermediates | Mitochondria | Gibson et al. (2000) |
| Pantothenate | Coenzyme-A (CoA) | TCA intermediates | Mitochondria and cytosol | Plesofsky-Vig (1996) |

[a] Details of iron requirements are discussed in the text.

Iron deficiency is a major cause of heme deficiency. Heme deficiency in non-erythroid tissues causes a loss of complex IV and oxidative stress (Atamna et al., 2001). It was once presumed that iron deficiency exerts most of its deleterious effects only if anemia was present. It is now clear that many organs show morphologic, physiologic, and biochemical changes before hemoglobin changes (Baranano et al., 2002), since iron is preferentially used for hemoglobin biosynthesis, even when the iron supply does not meet the body's total demand. Under conditions of iron deficiency, iron decreases differentially among organs in the body. Hepatic iron is lost first, followed by myocardial and CNS iron (Georgieff et al., 1990). Erythroid cells have the higher affinity for iron, which preserves iron under conditions of scarcity for hemoglobin biosynthesis (de Deungria et al., 2000). In myocardial cells, iron is preferentially used to synthesize cytochrome *c*, rather than produce myoglobin (Guiang et al., 1997), while cytochrome *c* decreases directly with the concentration of brain iron. A new model for iron deficiency has emerged in which iron deficiency differentially influences erythroid vs. non-erythroid tissues (Stoltzfus, 2001).

Prioritizing iron for erythroid tissue, puts the non-erythroid tissues at risk of heme deficiency even before signs of anemia become evident (de Deungria et al., 2000; Lozoff et al., 2000). Therefore a marker based on heme is needed to evaluate tissue status of iron. Leukocyte heme may be potential candidate.

A condition of iron deficiency in the absence of anemia has also been shown in women. Such deficiency decreases aerobic capacity and physical performance; parameters improved by iron supplementation (Brutsaert et al., 2003). Iron deficiency also occurs in the elderly (Freedman, 1987) and associated with cognitive decline (Milward et al., 1999). Surprisingly little is known about the effects of iron deficiency on brain dysfunction in the elderly.

Iron deficiency causes retardation in the development of the central nervous system, therefore impairs cognitive function in children, and impacts the morphology and the physiology of brain (Hurtado et al., 1999). Prenatal iron deficiency causes loss of complex IV in selected regions in the brain of rats (de Deungria et al., 2000), while iron deficiency at in vitro models causes a decrease in ferrochelatase (Taketani et al., 2000). Walter et al have demonstrated that iron deficiency damages mitochondria from rat liver and causes oxidant release (Walter et al., 2002). Thus, we believe that iron deficiency causes heme deficiency, leading to loss of complex IV, oxidant production, and decreased mitochondrial efficiency, which is likely to harm the developing brain. Iron deficiency at the early stages of the brain development may appear without clinical symptoms, although it may contribute to age-related disorders in the brain later in life (de Deungria et al., 2000).

## 7. Conclusions

The dependence of iron metabolism on mitochondria for the synthesis of heme (and iron–sulfur clusters) highlights the possible implication of these prosthetic groups, not only in disturbed iron homeostasis, but also in the mitochondrial decay of ageing and age-related diseases. The available research on heme suggests a larger role of heme in cell metabolism, in ageing, and age-related disorders. Clear dependence of heme production on nutritional and environmental factors is of interest since these factors can be controlled by intervention to optimize heme and prevent disturbed heme and iron metabolism with age. Keeping adequate

level of heme and heme-*a* could contribute to preventing age-related iron accumulation and mitochondrial decay, and delay oxidative damage.

The mitochondria appear susceptible to disturbed heme metabolism (Schipper et al., 2002). Heme is released from FC to the matrix of the mitochondria (Ponka, 1999). The advantage of locating heme biosynthesis in the mitochondria could be in part the need to co-localize the biosynthesis of the iron prosthetic groups (i.e. iron–sulfur cluster or heme) with the site of the biosynthesis of specific subunits of the electron transport chain (ETC), which anchor and use these prosthetic groups. Indeed most of the protein subunits of the ETC, which are critical for the actual electron transfer reactions, are also synthesized in the mitochondrial matrix by the mitochondrial genome. This co-localization should increase the metabolic efficiency of mitochondria by making heme available for the assembly of ETC complexes.

Another possible advantage of having iron metabolism to occur in the mitochondria is that some of the heme produced may serve as messenger molecules, which are exported from the mitochondrial matrix to the cytoplasm to become cytosolic uncommitted (free) heme that interacts with HRMs in specific cytosolic factors. An optimal level of uncommitted heme in the cytoplasm would indicate to nuclear DNA an adequate ETC and therefore functional mitochondria.

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