LONP1-DEPENDENT BREAKDOWN OF MITOCHONDRIAL 5-AMINOLEVULINIC ACID SYNTHASE PROTEIN BY HEME IN HUMAN LIVER CELLS
Qing Tian1*, Ting Li1*, Weihong Hou1, Jianyu Zheng1, Laura W. Schrum1,2, Herbert L. Bonkovsky1,2,3,4

1Liver, Digestive and Metabolic Disorders Laboratory and The Liver-Biliary-Pancreatic Center, Carolinas Medical Center, Charlotte, NC, 2Department of Biology, The University of North Carolina at Charlotte, Charlotte, NC, 3Department of Medicine, The University of Connecticut Health Center, Farmington, CT and 4Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC.

Running head: Heme-mediated breakdown of human mitochondrial ALAS-1

Address correspondence to: Herbert L. Bonkovsky, MD, Vice President of Research, Cannon Research Center Suite 201, Carolinas Medical Center, 1000 Blythe Blvd., Charlotte, NC 28203. E-mail: herbert.bonkovsky@carolinashealthcare.org; Fax: 704-355-7648.

Keywords: ALAS-1, heme, mitochondria, hepatocyte, HepG2, Huh-7
5-aminolevulinic acid synthase (ALAS-1) is the first and rate controlling enzyme that controls cellular heme biosynthesis. Negative feedback regulation of ALAS-1 by the end product heme is well documented and provides the foundation for heme treatment of acute porphyrias, a group of diseases caused by genetic defects in the heme biosynthesis pathway and exacerbated by controlled up-regulation of ALAS-1. Heme is known to affect ALAS-1 activity by repressing gene transcription, accelerating mRNA degradation, and impeding pre-ALAS-1 mitochondrial translocation. In the current study we examined the effect of heme on the rate of mature ALAS-1 protein turnover in human cells and tissues and explored the mediator involved in this new regulatory mechanism. We found that heme and other metalloporphyrins, such as CoPP and CrPP, decreased mitochondrial ALAS-1 protein through proteolysis. This degradative effect cannot be emulated by iron or free protoporphyrin, two major chemical components of the heme ring, and is independent of oxidative stress. Down-regulating the activity of mitochondrial LONP1, an ATP-dependent protease that controls the selective turnover of mitochondrial matrix proteins, with potent inhibitors and specific siRNA diminished the negative effect of heme on mitochondrial ALAS-1. Therefore our data support the existence of a conserved heme feedback regulatory mechanism that functions on the mature form of ALAS-1 protein through a mitochondrial proteolytic system.

The iron-protoporphyrin (heme) macrocycle is a primordial molecule that carries out a myriad of essential functions upon which most aerobic life on earth depends. The essential functions of heme in the transport of oxygen and carbon dioxide in the blood (hemoglobin) and in diverse other tissues (myoglobin, neuroglobin) are well known, as is its essential role in catalyzing and accelerating numerous redox reactions, as an essential prosthetic group for cytochromes, peroxidases, and oxygenases. In most animals, heme is synthesized from glycine and succinyl CoA in a complex pathway involving eight enzymes. The first and normally rate-controlling step is carried out by the mitochondrial enzyme 5-aminolevulinic acid (ALA) synthase. The ubiquitous, housekeeping isoform of ALA synthase is ALA synthase-1 (ALAS-1), levels of which can be increased remarkably by numerous drugs and chemicals through interactions with several nuclear receptors (e.g., CAR, PXR, RXR), which form heterodimers and act as positive transcription factors for the ALAS-1 gene, as well as those of cytochromes P-450, the hemoproteins chiefly responsible for phase I metabolism of numerous endogenous intermediates and xenobiotics (1-5). In contrast, heme, the end-product of the synthetic pathway, exerts potent down-regulation of expression of ALAS-1 in most tissues, an example of negative feedback repression (1,6). Glucose and other metabolizable sugars also down-regulate the expression of ALAS-1, acting chiefly through a transcriptional co-activator, PGC-1α (7).

Uncontrolled up-regulation of ALAS-1 is a biochemical hallmark and sine qua non of acute attacks of hepatic porphyrias, a group of largely inherited disorders in which variable degrees of deficiency in an enzyme of normal hepatic heme synthesis distal to ALAS-1 (e.g., ALA dehydratase, porphobilinogen deaminase, coproporphyrinogen oxidase, or protoporphyrinogen oxidase). Such deficiency, especially when coupled with another factor that increases the liver’s need for heme (e.g., for induction of cytochromes P-450 [CYPs]) and/or the rate of hepatic heme breakdown (e.g., suicide substrates of CYPs), leads to de-repression of ALAS-1 and thus to marked increases in ALA, porphobilinogen, and other intermediates proximal to the site of the metabolic block in the heme synthetic pathway (1,8-9).

In view of the above, it is not surprising that glucose loading and intravenous infusions of heme, which both repress ALAS-1, and, in the case of heme, also restores the normal hepatic heme pools; hence, over the years these treatments remain the mainstays of therapy of acute porphyritic attacks (1,10-11).

We and others have shown that heme down-regulates expression of the hepatic ALAS-1 gene in several ways, including diminution of gene transcription (12-13) and enhancement of the breakdown of the mRNA (14-16). In addition, heme has been shown to block the uptake of pre-
ALAS-1 into mitochondria (17-18), where the translocation signal sequence on the pre-ALAS-1 protein is cleaved and processed into the mature form. This step is essential for activity of the synthase because succinyl-CoA is synthesized in and present in meaningful concentrations only within the mitochondria.

In a previous effort to further extend our understanding of ALAS-1 regulation by heme and non-heme metalloporphyrins, we examined the changes in the ALAS-1 protein level in response to exogenous metalloporphyrin treatment. Our data showed varied protein expression patterns. Heme and CoPP significantly decreased the amount of a protein with a molecular weight corresponding to the mature form of ALAS-1, while increasing the level of a higher molecular weight form that matched the size of the precursor protein (19). These results led us to speculate a new level of regulation by heme that functions on affecting the protein turnover rate. Recently, studies performed in rats provided evidence that heme is also capable of enhancing the rate of disappearance (presumably breakdown) of ALAS-1 in mitochondria (20). Because of the importance of the synthase in human health and disease (the acute porphyrias), and in view of the manifold and still unfolding roles of heme in human biology and pathophysiology, we have assessed the effects of heme and other selected metalloporphyrins on the stability of ALAS-1 in mitochondria from human liver cells. We also have explored the mechanisms whereby heme leads to decreased levels of ALAS-1 in mitochondria.

**Experimental Procedures**

**Chemicals and Reagents-** Protoporphyrin IX (PP), Fe protoporphyrin (heme), Co protoporphyrin (CoPP), Cr protoporphyrin (CrPP), and Mn protoporphyrin (MnPP) were purchased from Frontier Scientific (Logan, UT). Dimethyl sulfoxide (DMSO) was purchased from Fisher Biotech (Fair Lawn, NJ). Rabbit anti-ALAS-1 polyclonal antibody, and mouse anti-VDAC1/Porin monoclonal antibody were from Abcam (Cambridge, MA), Iron (III) chloride (FeCl3), 4,6-dioxoheptanoic acid (DHA), butylated hydroxytoluene (BHT), tert-butyl hydroperoxide solution (tBuOOH), Sucrose, Tween-20, 4-Morpholinepropanesulfonic acid (MOPS), CA-074 methyl ester, epoxomicin, rabbit anti-LONP1 polyclonal antibody and protease inhibitor cocktail (P8340) were from Sigma-Aldrich (St. Louis, MO), rabbit anti-calnexin polyclonal antibody, goat anti-human GAPDH polyclonal antibody, mouse anti-rabbit IgG (ALAS-1, LONP1, and calnexin), goat anti-mouse IgG (VDAC1/Porin), and donkey anti-goat IgG (GAPDH) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). BCA protein assay reagent was from Pierce (Rockford, IL). ECL-Plus was purchased from Amersham Biosciences Corp (Piscataway, NJ). CA-074 Me, MG-262 and InnoZyme Cathepsin B Activity Assay Kit were from EMD Chemicals Inc. (Gibbstown, NJ). 20S Proteasome Assay Kit was from Cayman Chemical Company (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), Phosphate Buffered Saline (PBS) pH 7.4 (IX), Penicillin-Streptomycin, Fetal Bovine Serum (FBS) and trypsin were from Invitrogen Inc. (Carlsbad, CA). 7.5% gradient SDS-PAGE gels, nonfat dry milk and ImmunBlot PVDF membranes were purchased from Bio-Rad (Hercules, CA).

**Cell-line and Culture Conditions-** Huh-7, the human hepatocellular carcinoma cell line, was purchased from the Japan Health Research Resources Bank (Osaka, Japan). HepG2, another human liver hepatocellular carcinoma cell line, was obtained from American Type Culture Collection (Manassas, VA). The Huh-7.5 cell line was from Apath LLC (St, Louis, MO). Huh-7.5 is a highly permissive, alpha interferon-cured Huh-7 human hepatocellular carcinoma cell line derivative. All cells were maintained in DMEM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% (v/v) FBS. All cells were maintained in a humidified atmosphere of 95% room air and 5% CO2 at 37 ºC.

**Human liver tissue-** Liver biopsies were obtained from subjects without known liver disease who were undergoing laparoscopic upper abdominal surgery for other therapeutic indications (e.g., cholecystectomy, gastric-bypass surgery). Wedge biopsies (~1g) were obtained and placed immediately into buffer (250mM sucrose, 5mM MOPS, pH 7.2), put on ice, and transported to the laboratory. The protocol for obtaining human liver samples was received and approved by the Institutional Review Board of Carolinas Medical Center.
**siRNA Transfection-** A smart pool of siRNAs, was purchased from Dharmacon (Lafayette, CO). Transfections of human LONP1 siRNAs and the non-specific scrambles (control siRNAs) were performed with Lipofectamine™ 2000 from Invitrogen (Carlsbad, CA) as described previously (21). Huh-7 cells were plated in 24-well plates one day prior to transfection and transfected at 70–80% confluence. Cells were exposed to transfection medium for 48 h with siRNAs before harvesting.

**Cathepsin B Activity-** Cathepsin B activity was measured using an InnoZyme Cathepsin B Activity Assay Kit (EMD Chemicals Inc.). Manufacturer’s protocol was followed.

**20S Proteasomal Activity-** The 20S proteasome activity was determined in cell or mitochondrial extracts using 20S Proteasome Assay Kit (Cayman Chemical Company) as described by the manufacturer. The assay is based on the detection of the fluorophore 7-Amino-4-methylcoumarin (AMC). SUC-LLVY-AMC was used as substrate in this assay. The fluorescence of AMC is quenched by LLVY, upon cleavage into two separate fragments by 20S proteasome, the fluorescence of AMC is recovered, and can be quantified. Therefore, relative fluorescence units (RFU) of AMC is a measurement of 20S proteasome activity (excitation =360 nm; emission = 480nm).

**Isolation of Mitochondria and Electron Microscopy-** Harvested cells or fresh human liver tissue were homogenized gently in the buffer that contained 250 mM Sucrose and 20 mM MOPS (pH 7.2) on ice, then spun down by conventional differential centrifugation with a final spin at 8,600 xg. The mitochondria were suspended in 250 mM sucrose and 5 mM MOPS (pH 7.2). Mitochondrial protein was determined using the Pierce Coomassie protein assay reagent kit. The mitochondria-enriched pellets, prepared by differential centrifugation, were fixed in 4% glutaraldehyde, stained with OsO₄, sectioned at 1 micron, and examined by electron microscopy. Representative fields were photographed.

**Western Immunoblotting Analysis-** Protein preparations and Western immunoblotting were carried out as previously described (19). In brief, total proteins (75 µg) were separated on 7.5% gradient SDS-PAGE gels. After electrophoretic transfer onto ImmunBlot PVDF membranes, the membranes were blocked for 1 hour in PBS containing 5% nonfat dry milk and 0.1% Tween-20, and then incubated overnight with primary antibody at 4 °C. The dilutions of the primary antibodies were as follows: 1:5000 for anti-ALAS1, 1:2000 for VDAC1/Portin, 1:1000 for anti-LONP1, 1:1000 for anti-calnexin and 1:1000 for anti-GAPDH. Membranes were then incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies (dilution 1:10,000). Finally, the bound antibodies were visualized with the ECL-Plus chemiluminescence system according to the manufacturer’s protocol (Amersham, Piscataway, NJ). A Kodak 1DV3.6 computer-based imaging system (Eastman-Kodak, Rochester, NY) was used to measure the relative optical density of each specific band obtained after Western immunoblotting analysis. Data are expressed as percentage of the vehicle control (DMSO).

**Statistical Analysis-** Experiments were repeated a minimum of three times with similar results. All experiments included at least triplicate samples for each treatment group. Representative results from a single experiment are presented. Initial inspection showed that the results were normally distributed. Therefore, parametric statistical procedures were used. Statistical analyses of data were performed with JMP 6.0.3 software (SAS Institute, Cary, NC). Student’s t-test for comparisons of two means and analysis-of-variance (F statistics) for comparisons of more than two, with pair-wise comparisons by the Kruskal-Wallis test were performed when appropriate. Values of P <0.05 were considered significant.

**RESULTS**

**Mitochondrially-enriched Subcellular Fractions-** Transmission electron micrographs of the subcellular fractions enriched for mitochondria revealed abundant, intact mitochondria, with numerous cristae, as well as circular membrane-bound structures of various sizes. (Fig 1A). These fractions contained VDAC/Portin protein and the lower MW [68kDa] form of ALAS-1 but not the higher-MW pre-ALAS-1 (Fig 1B). They contained no detectable GAPDH (a marker of soluble cytosolic protein), nor calnexin (a marker of endoplasmic reticulum/microsomes).
Effect of Heme on Human Mitochondrial ALA synthase-1—Compared to vehicle (DMSO) alone, addition of heme (0.1-10µM) to mitochondrially-enriched fractions led to a temperature- and time-dependent decrease in detectable ALAS-1 (data not shown). The effects were maximal at 1µM exogenous heme added and increased progressively with length of incubation (0-8 h) (Fig. 2A). The effects observed at 30°C, the temperature routinely used, were abolished when incubations were performed at 0°C (results not shown). Effects of heme were similar when mitochondrially-enriched fractions of Huh-7, Huh-7.5 or HepG2 homogenates were compared (Fig. 2B). The half-life of ALAS-1 protein was 8.7 h in the absence of heme (DMSO as vehicle) but only 3.4 h (2.5-fold decrease) in the presence of heme 1µM (Fig. 2C). Depletion of endogenous heme using a heme synthetic inhibitor, 4,6-dioxoheptanoic acid (DHA), a potent inhibitor of heme synthesis, increased ALAS-1 protein stability (to 57.1 h). Additionally, heme also markedly decreased ALAS-1 protein levels in mitochondria isolated from human liver biopsies (Fig. 2D).

Effect of Iron, Free Protoporphyrin, Oxidative Stress and Non-heme Metalloporphyrins on ALAS-1 Expression—Heme is a porphyrin ring coordinated with an iron molecule. To further dissect the mechanism(s) of action of heme on mitochondrial ALAS-1, we also tested the effect of iron and free protoporphyrin on the fate of ALAS-1; neither affected expression, suggesting the requirement of an intact heme macrocycle for ALAS-1 regulation (Fig. 3A).

Mitochondria harbor cellular respiration machinery that is a major source of intracellular reactive oxygen species (ROS). Free cellular heme as the main carrier of redox-active iron can synergize with mitochondria-derived ROS and further promote oxidative stress. To evaluate whether the pro-oxidant property of heme contributes to the observed ALAS-1 down-regulation, we treated mitochondrial preparations with tBuOOH, an oxidant, and BHT, an antioxidant, in the presence or absence of heme. tBuOOH treatment resulted in decreased ALAS-1 expression (Fig. 3B), while BHT alone slightly increased ALAS-1 half-life (Fig. 3C), implying that redox regulation of ALAS-1 may exist. However, BHT did not affect heme-induced ALAS-1 down-regulation (Fig. 3D), strongly suggesting that heme exerts its effect on ALAS-1 through a mechanism independent of oxidative stress.

Addition of other metalloporphyrins (1µM), especially cobalt or chromium protoporphyrin, also led to a decrease in ALAS-1 protein in mitochondrially-enriched fractions from Huh-7 cells or human liver tissue (Fig. 3E).

Effect of Protease Inhibitors on ALAS-1—A protease inhibitor cocktail consisting of aprotinin, leupeptin, bestatin, pepstatin A, and E-64, inhibiting a wide-spectrum of protease activity that might be present in the mitochondrial preparations, was used to determine if protein degradation accounts for reduced ALAS-1 protein expression in mitochondria. The cocktail increased the amount of mitochondrial ALAS-1 in the absence of heme and also minimized the down-regulating effect of heme on ALAS-1 (Fig. 4A), suggesting that a proteolytic event is involved in mitochondrial regulation of ALAS-1.

CA-074 Me, a selective inhibitor of cathepsin B, produced a profound and durable inhibition of activity of this lysosomal enzyme (supplemental Fig. S1). Nevertheless, even in the presence of such inhibition, heme continued to exert its down-regulatory effect on ALAS-1 protein (Fig. 4B).

MG-262 is a potent boron-containing inhibitor of Lon protease. Addition of MG-262 (10µM), either 4h prior to, or concurrent with, the addition of heme (1µM), led to a complete abrogation of the heme effect to accelerate the disappearance of ALAS-1 protein (Fig 4C). In fact, under both heme and no heme conditions, MG-262 slightly increased the level of ALAS-1 protein compared to null MG-262 treatment, suggesting that the inhibitory function of MG-262 stabilized ALAS-1. The greater selectivity of MG-262 for and the higher potency against 20S proteasome over LONP1 raises concern about the proteasomal involvement in this process. Indeed we detected residual proteasomal activity in our mitochondrial preparation (supplemental Fig. S2). Therefore, we used a more specific proteasome inhibitor, epoxomicin, which showed no effect on LONP1 activity (22). Epoxomicin, at the concentration that significantly decreased the proteasomal activity in our mitochondrial preparation (supplemental Fig. S2), did not affect
heme-mediated disappearance of ALAS-1 (Fig. 4D). To further establish the role of LONP1 protease in the heme-induced decrease in mitochondrial ALAS-1, we silenced the endogenous LONP1 protease gene by transfecting hepatocytes with LONP1 specific siRNA pools. As expected, the siRNAs markedly decreased levels of endogenous LONP1 protein (supplemental Fig. S3A) without significant reduction in the steady state protein level of ALAS-1 (Fig. 4E). LONP1 knockdown diminished heme-dependent down-regulation of ALAS-1 protein (Fig. 4E). siRNA transfection did not alter the 20S proteasomal activity (supplemental Fig. S3B), supporting the notion that proteasome was not responsible for the observed decrease of ALAS-1 by heme. In other experiments, we found that exogenous heme (1µM for 8 h) did not affect levels of LONP1 protein in mitochondrially-enriched fractions (supplemental Fig. S4).

DISCUSSION

Previous studies performed by our group showed that in cell lines of human hepatocellular carcinomas exogenous heme down-regulates the lower molecular weight band of human ALAS-1 protein (19), which corresponds to the size of the mature form ALAS-1 located in the mitochondria, suggesting a heme-induced mechanism that controls the fate of the functional form of ALAS-1. In the present study, we further investigated this possibility in isolated mitochondria where pre-ALAS-1 is processed into the mature protein and poised for initiating heme synthesis. Our data indicate that in both human cell lines and liver tissue heme significantly decreases the half-life of mitochondrial ALAS-1, whereas in heme-depleted conditions, generated with DHA pretreatment, an inhibitor of the second enzyme of the heme biosynthesis pathway, mitochondrial ALAS-1 exhibited prolonged stability. Iron and protoporphyrin did not emulate the effect of heme, and addition of an antioxidant failed to diminish the decrease of ALAS-1.

Heme has been known to down-regulate ALAS-1 by a negative feedback loop that involves repression of transcription, acceleration of mRNA degradation, and impairment of mitochondrial import of the protein (1-2,7). In the current study we demonstrated an additional dimension of posttranslational regulation of ALAS-1, adding to the complexity of the heme control of ALAS-1 function. One apparent advantage of such regulation is the control of ALAS-1 activity inside mitochondria, allowing for very rapid response of the heme biosynthetic pathway to intramitochondrial levels of heme. Similar findings in rat liver mitochondria were reported recently (20), suggesting that direct mitochondrial control of ALAS-1 by heme is a conserved regulatory mechanism across species.

Treating isolated mitochondria with a protease inhibitor reversed the negative effect of heme on ALAS-1 protein, suggesting that a proteolytic process is involved. We excluded the possible involvement of proteasomal activity in this process by using the proteasome specific inhibitor epoxomicin. Epoxomicin exerted no effect on heme-mediated degradation of ALAS-1. The turnover of mitochondrial proteins is usually determined by two pathways: 1) lysosomal degradation via autophagy, and 2) mitochondria-specific protein quality control system (23-24). Our results with a cathepsin B inhibitor indicated that the lysosomal system made minimum contribution to heme-induced ALAS-1 turnover, suggesting that a mitochondrial protease carried out the degradation. It is well-established that mitochondrially located proteins face constant oxidative challenges due to the presence of inevitable ROS by-products of aerobic respiration; such stress-induced protein damage and aggregation pose risks to normal biological functions. Therefore, monitoring for and scavenging damaged proteins are needed on a continuous basis. However, compared to the cytosolic protein quality control system, mitochondrial proteases and chaperones are less well-characterized. Our data suggest that LONP1, a nuclearly-encoded and mitochondrially-located stress responsive protease, is involved in heme-mediated ALAS-1 turnover. Mammalian Lon1 protease is a homologue of bacterial protease La and yeast PIM1 and has been implicated throughout evolution in the degradation of stress-damaged mitochondrial enzymes (25). A second ATP-dependent matrix protease identified to date is ClpXP. Thus far no physiologically significant substrates have been assigned to mammalian ClpXP. We do not exclude the possibility that
other mitochondrial protease systems are also involved in heme-ALAS-1 regulation.

The molecular mechanism of heme-mediated mitochondrial ALAS-1 degradation also calls for further investigation. How ALAS-1 in presence of heme overload is marked for degradation remains unanswered. The lack of effect of iron or free porphyrin and the observation that the down-regulating activity of heme is separate from redox regulation point to a mechanism by which the entire heme macrocycle is required as a signaling molecule. Heme as a prosthetic group affects the function and fate of many proteins. Noteworthy examples include the important mammalian transcription repressor Bach1 and yeast transcription activator HAP1 (26-27). ALAS-1 has three CP motifs known as heme regulatory motifs, one in the N-terminus of the mature protein and two in the leader sequence of the preprotein. The functional role of these CP motifs in ALAS-1 has been studied in respect to heme regulation of mitochondrial translocation (17-18). Similar CP motifs have been identified in Bach1 and HAP1 and shown to regulate the activity of these proteins. We plan to examine whether the CP motif functions as a heme sensor and mediates the heme effect on mitochondrial ALAS-1.

Another aspect that needs to be taken into consideration involves mitochondrial matrix protein processing. Like most mitochondrial matrix proteins ALAS-1 is synthesized on the rough endoplasmic reticulum and subsequently transferred across the outer- and inner-membranes of mitochondria, a process requiring additional processing by peptidases and protection by chaperones for proper folding. Whether heme regulates pre-sequence processing and how that would affect ALAS-1 stability and function are also in need of further examination. Our present experimental system was not designed to address these issues and future research is warranted.

Heme infusion is the standard therapy for acute porphyrias but its effect is often short-lived due to the activation of heme oxygenase-1 and sometimes associated with undesirable side effects. Indeed, recent studies in our lab show that heme treatment increases mRNA expression of several pro-inflammatory genes and stress response genes (Bonkovsky, unpublished). Many synthetic metalloporphyrins are structurally related to heme but display varied activity towards activating heme oxygenase and inducing adverse reactions. Non-heme metalloporphyrins, therefore, are considered potential alternatives for the effective treatment of porphyria. We also examined the effect of other metalloporphyrins on ALAS-1 protein expression; we found that three transition metal-complexed porphyrins, namely CoPP, CrPP, MnPP, also down-regulate mitochondrial ALAS-1 to various extents. CoPP and CrPP have been shown to regulate ALAS-1 mRNA levels (19). A better understanding of how non-heme metalloporphyrins work is essential for developing new therapeutic strategies.

REFERENCES


**FOOTNOTES**

This work was supported by a grant (5R01DK38825) from NIH to Dr. Herbert L. Bonkovsky. We thank Dr. Alexander Panov for advice regarding methods for preparation of mitochondrially-enriched fractions and Dr. Sriparna Ghosh for providing calnexin antibody. Additionally, we thank Dr. Keith Gersin and Amanda Balasco who helped to provide human liver tissue and Patsy McCoy and David Radoff for their assistance with electron microscopy. We are also grateful to Ms. Ashley Lakner for her critical reading of and suggestions regarding the manuscript.

List of Abbreviations: ALA, 5-aminolevulinic acid; ALAS-1, 5-ALA synthase-1; BHT, butylated hydroxytoluene; CoPP, cobalt protoporphyrin; CrPP, chromium protoporphyrin; DHA, 4,6-dioxoheptanoic acid; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FeCl3, iron chloride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LONP1, Lon peptidase 1; MG-262, Z-Leu-Leu-Leu-B (OH); MnPP, manganese protoporphyrin; MOPS, morpholinepropanesulfonic acid; OsO4, osmium tetroxide; PP, Protoporphyrin IX; ROS, reactive oxygen species; tBuOOH, tert-butyl hydroperoxide; VDAC, voltage-dependent anion channel.

* These authors contributed equally.
FIGURE LEGENDS

Fig. 1. Mitochondrial preparations from human hepatocytes and cell lines. HepG2 cells were cultured, harvested, homogenized, and mitochondrially-enriched fractions prepared as described in Materials and Methods. Mitochondrial-enriched pellets (75µg protein) were visualized by (A) transmission electron microscopy (panel 1 = 13,500x; panels 2 and 3 = 92,000x) and (B) total cell and mitochondrial extracts from human liver biopsies were subjected to Western immunoblotting analysis for ALAS-1 protein expression. VDAC1/porin, Calnexin, and GAPDH expression served as mitochondrial, ER/microsome, and total lysate controls, respectively.

Fig. 2. Effects of heme on ALAS-1 protein expression in mitochondria from human hepatocytes and cell lines. (A) Mitochondrial fractions (75µg protein) were incubated at 30°C with heme (1µM) for indicated time period to examine the time-course of the heme effect. (B) Mitochondria from various liver cell lines were incubated with buffer, DMSO, or heme for 8 h and ALAS-1 protein levels were assessed by Western immunoblotting analysis. (C) ALAS-1 protein half-life was determined by quantifying band intensities of remaining ALAS-1 protein normalized to VDAC1/porin and plotted on semi-logarithmic graph. Half-lives were 8.7 h, 3.4 h or 51.7 h following exposure of cells to DMSO, heme or DHA, respectively. (D) The effect of heme (1µM) on ALAS-1 was examined in mitochondria from human liver biopsies.

Fig. 3. Effect of iron, free protoporphyrin, oxidative stress and non-heme synthetic metalloporphyrins on ALAS-1 expression. (A) Mitochondria from Huh-7 cells were incubated with FeCl3 (1µM), free protoporphyrin IX (PP, 1µM), or heme (1µM) or DMSO for 8 h and Western immunoblotting analysis performed for ALAS-1 expression. (B) Mitochondrial preparations were treated with tBuOOH (1µM), an oxidant for 0, 1, 2, 4 or 8 h and ALAS-1 expression determined by Western immunoblotting analysis. (C and D) Similar experiments were performed with BHT (10µM), an anti-oxidant, in the presence or absence of heme (1µM). (E) Incubation with other metalloporphyrins (1µM), including CoPP, CrPP or MnPP, was also performed and ALAS-1 protein expression determined by Western immunoblotting analysis. Upper panel - mitochondria from Huh-7 cells; bottom panel – mitochondria from human liver biopsies.

Fig. 4. Effect of protease inhibitors and siRNAs on ALAS-1 protein expression. (A) Mitochondrial extracts from Huh-7 cells (75µg total protein) were incubated (30°C) with DMSO, or heme (1µM) with or without a protease inhibitor cocktail for 24 h. ALAS-1 protein expression was determined by Western immunoblotting analysis. (B) Similar studies were performed using the selective cathepsin B inhibitor, CA-074 Me. Mitochondrial extracts (75µg total protein) were pre-treated for 4 h with 1µM of CA-074 Me, then incubated (30°C) with DMSO, heme (1µM) with or without a protease inhibitor cocktail for 8 h. ALAS-1 protein expression was determined by Western immunoblotting analysis. (C) MG-262 inhibits heme-mediated down-regulation of ALAS-1. Mitochondrial extracts (75µg total protein) were pre-incubated with or without MG-262 (10µM) for 4 h, then subsequently incubated (30°C) with heme or DMSO for 8 h. ALAS-1 protein expression was determined by Western immunoblotting analysis as described in the Experimental Procedures. (D) The effect of Epoxomicin on heme-induced mitochondrial ALAS-1 degradation in mitochondrial extracts. Mitochondrial extracts (75µg total protein) were pre-incubated with or without epoxomicin (1µM) for 4 h, then subsequently incubated (30°C) with heme or DMSO for 8 h. ALAS-1 protein expression was assayed by Western immunoblotting analysis. (E) LONP1 protease was silenced by transfecting Huh-7 cells with siRNA specific for the LONP1 gene (25nM). Mitochondrial fractions were prepared after transfection and incubated (30°C) with DMSO or heme (1µM in DMSO) for 8 h. ALAS-1/LONP1 protein expression was determined by Western blot analysis.
A.

1

2

3

B.

ALAS-1

VDAC1/Porin

Calnexin

GAPDH

Total Lysate

Mitochondria
Figure 2. Tian et al., JBC
Figure 2. Tian et al., JBC

B.

ALAS-1
VDAC1/Porin
ALAS-1
VDAC1/Porin
ALAS-1
VDAC1/Porin

Huh-7
Huh-7.5
HepG2

None DMSO Heme

Relative Mitochondrial ALAS-1 Protein

Huh-7
Huh-7.5
HepG2

* p< 0.01
Figure 2. Tian et al., JBC
Figure 2. Tian et al., JBC

D.

![Western Blot Image]

**Relative Mitochondrial ALAS-1 Protein**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALAS-1</th>
<th>VDAC1/Porin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heme</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.001*
Figure 3. Tian et al., JBC

A.

**ALAS-1**

**VDAC1/Porin**

<table>
<thead>
<tr>
<th></th>
<th>ALAS-1</th>
<th>VDAC1/Porin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeCl₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heme</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative Mitochondrial ALAS-1 Protein

![Graph showing relative mitochondrial ALAS-1 protein levels with asterisk indicating p < 0.05](http://www.jbc.org/)

* p < 0.05
Figure 3. Tian et al., JBC

B. tBuOOH

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ALAS-1</th>
<th>VDAC1/Porin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. BHT

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ALAS-1</th>
<th>VDAC1/Porin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D. BHT + Heme

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ALAS-1</th>
<th>VDAC1/Porin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Tian et al., JBC

E.

<table>
<thead>
<tr>
<th>None</th>
<th>DMSO</th>
<th>Heme</th>
<th>CoPP</th>
<th>CrPP</th>
<th>MnPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS-1</td>
<td>VDAC1/Porin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>None</th>
<th>DMSO</th>
<th>Heme</th>
<th>CoPP</th>
<th>CrPP</th>
<th>MnPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS-1</td>
<td>VDAC1/Porin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Tian et al., JBC

A.

**ALAS-1**

**VDAC1/Porin**

<table>
<thead>
<tr>
<th>None</th>
<th>DMSO</th>
<th>Heme</th>
<th>Inhibitor</th>
<th>Inhibitor + Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Mitochondrial ALAS-1 Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.001*
Figure 4. Tian et al., JBC
C.

ALAS-1

VDAC1/Porin

DMSO
Heme
MG-262

0 h 8 h

Relative Mitochondrial
ALAS-1 Protein

DMSO
Heme
MG-262
Figure 4. Tian et al., JBC

D.

ALAS-1

VDAC1/Porin

DMSO

Heme

Epoxomicin

Relative Mitochondrial ALAS-1 Protein

0.2
0.4
0.6
0.8
1.0
1.2

DMSO

Heme

Epoxomicin
Figure 4. Tian et al., JBC

E.

![Image of Figure 4 with LONP1, ALAS-1, and VDAC1/Porin bands under different treatment conditions (Control siRNA, Heme, LONP1 siRNA).]

![Graph showing relative mitochondrial ALAS-1/LONP1 protein levels under different conditions.]
LONP1-dependent breakdown of mitochondrial 5-aminolevulinic acid synthase protein by heme in human liver cells
Qing Tian, Ting Li, Weihong Hou, Jianyu Zheng, Laura W. Schrum and Herbert L. Bonkovsky

J. Biol. Chem. published online June 9, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.215772

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/06/09/M110.215772.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2011/06/09/jbc.M110.215772.full.html#ref-list-1