

# Lon Peptidase 1 (LONP1)-dependent Breakdown of Mitochondrial 5-Aminolevulinic Acid Synthase Protein by Heme in Human Liver Cells<sup>\*[5]</sup>

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5-Aminolevulinic acid synthase (ALAS-1) is the first 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 the activity of 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 synthase (ALAS).<sup>3</sup> The ubiquitous, housekeeping isoform of ALAS is ALAS-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, and 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-1 $\alpha$  (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 need of the liver for heme (*e.g.* for induction of cytochromes P-450) and/or the rate of hepatic heme breakdown (*e.g.* suicide substrates of cytochromes P-450), leads to derepression 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 porphyric attacks (1, 10, 11).

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<sup>3</sup> The abbreviations used are: ALAS-1, 5-aminolevulinic acid synthase-1; BHT, butylated hydroxytoluene; CoPP, cobalt protoporphyrin; CrPP, chromium protoporphyrin; DMSO, dimethyl sulfoxide; LONP1, Lon peptidase 1; MG-262, Z-Leu-Leu-Leu-B(OH)<sub>2</sub>; MnPP, manganese protoporphyrin; AMC, 7-amino-4-methylcoumarin; PP, protoporphyrin IX; VDAC, voltage-dependent anion channel.

We and others (12, 13) have shown that heme down-regulates expression of the hepatic *ALAS-1* gene in several ways, including diminution of gene transcription 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 cobalt protoporphyrin (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), iron protoporphyrin (heme), CoPP, chromium protoporphyrin (CrPP), and manganese 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 ( $\text{FeCl}_3$ ), 4,6-dioxoheptanoic acid, butylated hydroxytoluene (BHT), *tert*-butyl hydroperoxide solution, sucrose, Tween 20, MOPS, CA-074 methyl ester, epoxomicin, rabbit anti-LONP1 polyclonal antibody, and protease inhibitor mixture (P8340) were from Sigma-Aldrich; and 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 (Santa Cruz, CA). BCA protein assay reagent was from Pierce. ECL-Plus was purchased from Amersham Biosciences. MG-262 and InnoZyme Cathepsin B Activity Assay Kit were from EMD Chemicals (Gibbstown, NJ). The 20S Proteasome Assay Kit was from Cayman Chemical Company (Ann Arbor, MI). DMEM, PBS, pH 7.4 (1 $\times$ ), penicillin-streptomycin, FBS, and trypsin were from Invitrogen. 7.5% gradient

SDS-PAGE gels, nonfat dry milk, and ImmunBlot PVDF membranes were purchased from Bio-Rad.

**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 the 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  $\mu\text{g}/\text{ml}$  streptomycin, and 10% (v/v) FBS. All cells were maintained in a humidified atmosphere of 95% room air and 5%  $\text{CO}_2$  at 37  $^\circ\text{C}$ .

**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 and gastric-bypass surgery). Wedge biopsies (~1 g) were obtained and placed immediately into buffer (250 mM sucrose, 5 mM 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 SMARTpool of siRNAs was purchased from Dharmacon (Lafayette, CO). Transfections of human LONP1 siRNAs and the nonspecific scrambles (control siRNAs) were performed with Lipofectamine<sup>TM</sup> 2000 from Invitrogen as described previously (21). Huh-7 cells were plated in 24-well plates 1 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). The manufacturer's protocol was followed.

**20S Proteasomal Activity**—The 20S proteasome activity was determined in cell or mitochondrial extracts using the 20S Proteasome Assay Kit (Cayman Chemical) 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 the 20S proteasome, the fluorescence of AMC is recovered and can be quantified. Therefore, relative fluorescence units of AMC is a measurement of 20S proteasome activity (excitation, 360 nm; emission, 480 nm).

**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 and then were spun down by conventional differential centrifugation with a final spin at 8600  $\times g$ . 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 osmium tetroxide, sectioned at 1 micron, and examined by electron microscopy. Representative fields were photographed.

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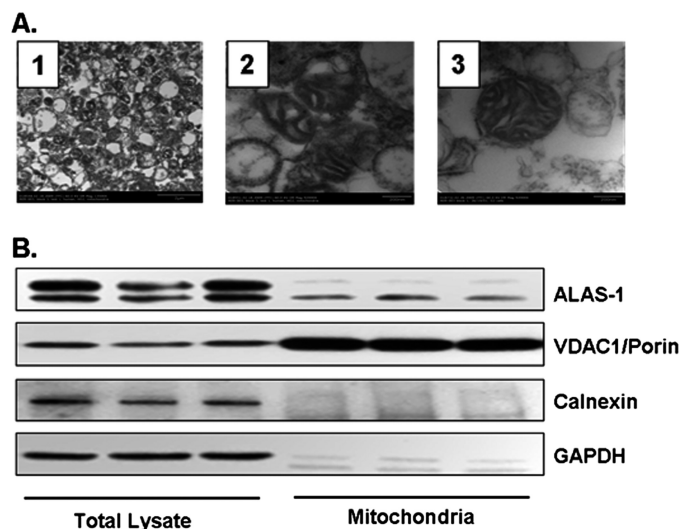
**Western Immunoblotting Analysis**—Protein preparations and Western immunoblotting were carried out as described previously (19). In brief, total proteins (75  $\mu\text{g}$ ) were separated on 7.5% gradient SDS-PAGE gels. After electrophoretic transfer onto Immobilon-P PVDF membranes, the membranes were blocked for 1 h 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-ALAS-1, 1:2000 for VDAC1/porin, 1:1000 for anti-LONP1, 1:1000 for anti-calnexin, and 1:1000 for anti-GAPDH. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution). Finally, the bound antibodies were visualized with the ECL-Plus chemiluminescence system according to the manufacturer's protocol (Amersham Biosciences). A Kodak 1D3.6 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 VDAC1/porin protein and the lower molecular mass (68 kDa) form of ALAS-1 but not the higher molecular mass pre-ALAS-1 (Fig. 1B). They contained no detectable GAPDH (a marker of soluble cytosolic protein) or calnexin (a marker of endoplasmic reticulum/microsomes).

**Effect of Heme on Human Mitochondrial ALAS-1**—Compared with vehicle (DMSO) alone, addition of heme (0.1–10  $\mu\text{M}$ ) to mitochondrially enriched fractions led to a temperature- and time-dependent decrease in detectable ALAS-1 (data not shown). The effects were maximal at 1  $\mu\text{M}$  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 1  $\mu\text{M}$  (Fig. 2C). Depletion of endogenous heme using a



**FIGURE 1. Mitochondrial preparations from human hepatocytes and cell lines.** HepG2 cells were cultured, harvested, homogenized, and mitochondrially enriched fractions prepared as described under "Experimental Procedures." Mitochondria-enriched pellets (75  $\mu\text{g}$  of protein) were visualized by transmission electron microscopy (panel 1 = 13,500 $\times$ ; panels 2 and 3 = 92,000 $\times$  (A)), and total cell and mitochondrial extracts from human liver biopsies were subjected to Western immunoblotting analysis for ALAS-1 protein expression (B). VDAC1/porin, calnexin, and GAPDH expression served as mitochondrial, endoplasmic reticulum/microsome, and total lysate controls, respectively.

heme synthetic inhibitor, 4,6-dioxoheptanoic acid, 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. Free cellular heme as the main carrier of redox-active iron can synergize with mitochondria-derived reactive oxygen species and further promote oxidative stress. To evaluate whether the prooxidant property of heme contributes to the observed ALAS-1 down-regulation, we treated mitochondrial preparations with *tert*-butyl hydroperoxide solution, an oxidant, and BHT, an antioxidant, in the presence or absence of heme. *Tert*-butyl hydroperoxide solution treatment resulted in decreased ALAS-1 expression (Fig. 3B), whereas 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 (1  $\mu\text{M}$ ), 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).

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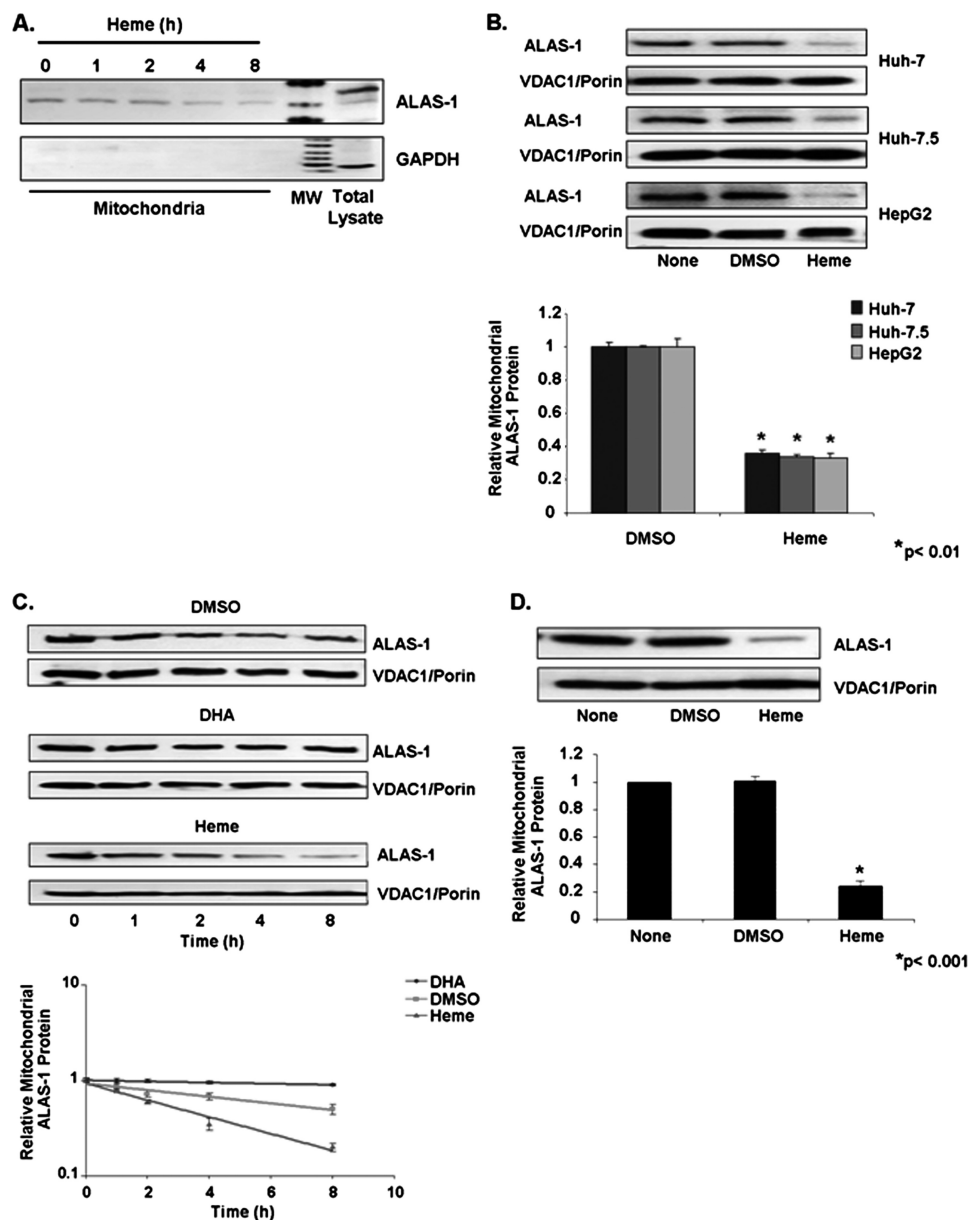


FIGURE 2. **Effects of heme on ALAS-1 protein expression in mitochondria from human hepatocytes and cell lines.** *A*, mitochondrial fractions (75  $\mu$ g of protein) were incubated at 30 °C with heme (1  $\mu$ M) for indicated time period to examine the time course of the heme effect. *MW*, molecular weight. *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, 3.4, or 51.7 h following exposure of cells to DMSO, heme, or 4,6-dioxoheptanoic acid (DHA), respectively. *D*, the effect of heme (1  $\mu$ M) on ALAS-1 was examined in mitochondria from human liver biopsies. Results of *bar graphs* are mean  $\pm$  SE.

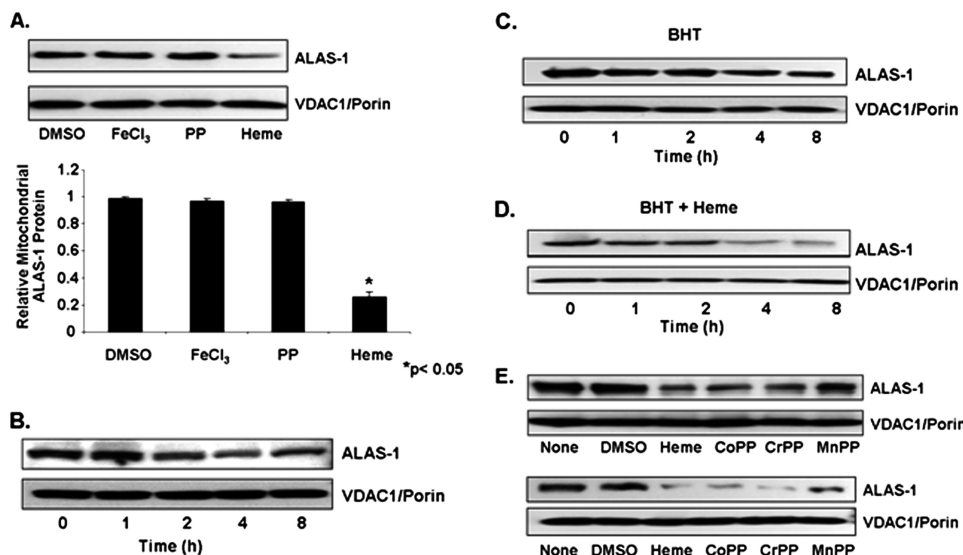
**Effect of Protease Inhibitors on ALAS-1**—A protease inhibitor mixture consisting of aprotinin, leupeptin, bestatin, pepstatin A, and E-64, inhibiting a wide spectrum of protease activity that might be present in the mitochondrial preparations, was used to determine whether protein degradation accounts for reduced ALAS-1 protein expression in mitochondria. The mixture 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 pres-

ence 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 (10  $\mu$ M), either 4 h prior to, or concurrent with, the addition of heme (1  $\mu$ M), 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 with 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

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**FIGURE 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 FeCl<sub>3</sub> (1 μM), free protoporphyrin IX (1 μM), or heme (1 μM) or DMSO for 8 h, and Western immunoblotting analysis was performed for ALAS-1 expression. Results of bar graph are mean ± SE. *B*, mitochondrial preparations were treated with *tert*-butyl hydroperoxide solution (*t*BuOOH; 1 μM), an oxidant for 0, 1, 2, 4, or 8 h, and ALAS-1 expression was determined by Western immunoblotting analysis. *C* and *D*, similar experiments were performed with BHT (10 μM), an antioxidant, in the presence or absence of heme (1 μM). *E*, incubation with other metalloporphyrins (1 μM), including CoPP, CrPP, or MnPP, was also performed, and ALAS-1 protein expression was determined by Western immunoblotting analysis. *Upper panel*, mitochondria from Huh-7 cells; *bottom panel*, mitochondria from human liver biopsies.

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 20 S 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 (1 μM for 8 h) did not affect levels of LONP1 protein in mitochondrially enriched fractions (supplemental Fig. S4).

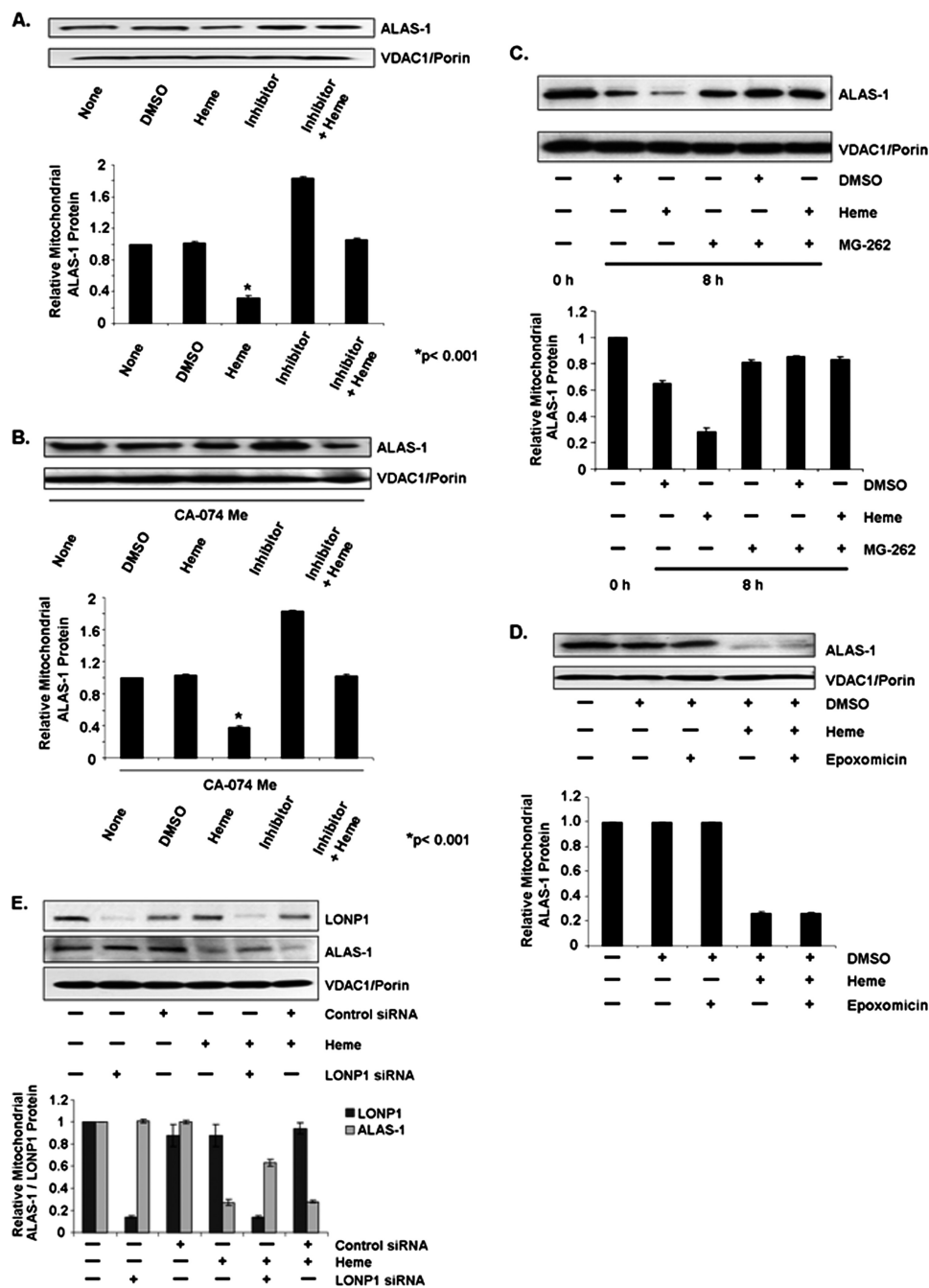
### DISCUSSION

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 4,6-dioxoheptanoic acid 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 mito-



**FIGURE 4. Effect of protease inhibitors and siRNAs on ALAS-1 protein expression.** *A*, mitochondrial extracts from Huh-7 cells (75 μg of total protein) were incubated (30 °C) with DMSO or heme (1 μM) with or without a protease inhibitor mixture for 8 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 (75 μg of total protein) were pretreated for 4 h with 1 μM of CA-074 Me, then incubated (30 °C) with DMSO, heme (1 μM) with or without a protease inhibitor mixture 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 (75 μg total protein) were preincubated with or without MG-262 (10 μM) 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 under "Experimental Procedures." *D*, the effect of epoxomicin on heme-induced mitochondrial ALAS-1 degradation in mitochondrial extracts. Mitochondrial extracts (75 μg total protein) were preincubated with or without epoxomicin (1 μM) for 4 h and 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 (25 nM). Mitochondrial fractions were prepared after transfection and incubated (30 °C) with DMSO or heme (1 μM in DMSO) for 8 h. ALAS-1/LONP1 protein expression was determined by Western blot analysis. Results of bar graphs are mean ± SE.

chondrial protease carried out the degradation. It is well established that mitochondrially located proteins face constant oxidative challenges due to the presence of inevitable reactive oxygen species 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 with the cytosolic protein quality control system, mitochondrial proteases and chaperones are less well characterized. Our data suggest that LONP1, a nuclear encoded and

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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 presequence 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 laboratory show that heme treatment increases mRNA expression of several proinflammatory genes and stress response genes.<sup>4</sup> Many synthetic metalloporphyrins are structurally related to heme but display varied activity toward 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, and 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.

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<sup>4</sup> H. L. Bonkovsky, unpublished observations.