Ambient Light Promotes Selective Subcellular Proteotoxicity after Endogenous and Exogenous Porphyrinogenic Stress*

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Background: Porphyrinogenic stress causes liver damage in association with lamin aggregation. Hepatic accumulation of protoporphyrin-IX (PP-IX) in erythropoietic protoporphyria (EPP) or X-linked-dominant protoporphyria (XLP) causes liver damage. Hepatocyte nuclear lamin aggregation is a sensitive marker for PP-IX-mediated liver injury. We tested the hypothesis that extracellular or intracellular porphyrinogenic stress causes compartment-selective proteotoxic damage and protein aggregation.

Results: Endogenously and exogenously stimulated protoporphyrin-IX accumulation leads to ambient light-triggered organelle-selective protein aggregation, ultrastructural alterations, ER stress, and proteasome inhibition.

Conclusion: External and internal porphyrinogenic stress cause compartment-selective proteotoxic damage and protein aggregation.

Significance: Subcellular compartment-specific and porphyrin-selective protein aggregation may be a hallmark injury mechanism in porphyrias.

Hepatic accumulation of protoporphyrin-IX (PP-IX) in erythropoietic protoporphyria (EPP) or X-linked-dominant protoporphyria (XLP) cause liver damage. Hepatocyte nuclear lamin aggregation is a sensitive marker for PP-IX-mediated liver injury. We tested the hypothesis that extracellular or intracellular porphyrinogenic stress causes damage to different subcellular compartments, in a light-triggered manner. Three hepatoma cell lines (HepG2, Hepa-1, and Huh-7) were treated with exogenous PP-IX (mimicking XLP extrahepatic protoporphyria) or with the iron chelator deferoxamine and the porphyrin precursor 5-aminolevulinic acid (ALA) (mimicking intracellular protoporphyrin accumulation in EPP). Exogenous PP-IX accumulated predominantly in the nuclear fraction and caused nuclear shape deformation and cytoplasmic vacuoles containing electron-dense particles, whereas ALA+deferoxamine treatment resulted in higher PP-IX in the cytoplasmic fraction. Protein aggregation in the nuclear and cytoplasmic fractions paralleled PP-IX levels and, in cell culture, the effects were exclusively ambient light-mediated. PP-IX and ALA caused proautophagic inhibition, whereas endoplasmic reticulum protein aggregation was more prominent in ALA-treated cells. The enhanced ALA-related toxicity is likely due to generation of additional porphyrin intermediates including uroporphyrin and coproporphyrin, based on HPLC analysis of cell lysates and the culture medium, as well as cell-free experiments with uroporphyrin/coproporphyrin. Mouse livers from drug-induced porphyria phenocopy the in vitro findings, and mass spectrometry of liver proteins isolated in light/dark conditions showed diminished (as compared with light-harvested) but detectable aggregation under dark-harvested conditions. Therefore, PP-IX leads to endoplasmic reticulum stress and proteasome inhibition in a manner that depends on the source of porphyrin buildup and light exposure. Porphyrin-mediated selective protein aggregation provides a potential mechanism for porphyria-associated tissue injury.

Protoporphyrin-IX (PP-IX) is the tetrathroprole biological precursor of heme. Heme biosynthesis occurs through a multistep enzymatic pathway and is spatially separated between the cytosol and mitochondria (1). In the mitochondrial lumen, 5-aminolevulinic acid (ALA) synthase initiates the synthetic pathway by combining L-glycine and succinyl Co-A to generate ALA, the first committed metabolite of the pathway (1). Once synthesized, ALA exits the mitochondria into the cytosol and is converted by ALA dehydratase to porphobilinogen, the monopyrrole subunit of the tetrapyrrole heme (2). Sequential condensation and deamination of four porphobilinogen moieties lead to the formation of the linear tetrapyrrole, hydroxymethylbilane (2). In the cytosol, hydroxymethylbilane is converted first to uroporphyrinogen/uroporphyrin, which is then converted to coproporphyrinogen/coproporphyrin and transported back into the mitochondria (2). In the penultimate step of the pathway, PP-IX is generated in the mitochondrial lumen by enzymatic oxidation of protoporphyrinogen IX (2). Ferrochelatase (Fech) then inserts Fe2+ in the tetrapyrrole ring of PP-IX to form functional heme (1). Porphyrins are bioactive toxic metabolites and, therefore, their biosynthesis is tightly regulated.

Porphyria is the accumulation of porphyrins due to inborn errors in heme biosynthesis. Mutations in each of the eight enzymes involved in the heme biosynthetic pathway are associated with various porphyria types, with PP-IX being the hallmark of porphyria cutanea tarda (PCT) and variegate porphyria (VP) (1). Porphyrinogenic stress such as sunlight, certain drugs, and metalloproteins can cause liver damage in porphyric patients (3). Therefore, porphyria is a relevant model to study the potential complications of porphyria.

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‡ The abbreviations used are: PP-IX, protoporphyrin-IX; Uro, uroporphyrin; Copro, coproporphyrin; EPP, erythropoietic protoporphyria; XLP, X-linked dominant protoporphyria; DCC, 3,5-dicarbethoxy-1,4-dihydrocollidine; ALA, 5-aminolevulinic acid; DFO, deferoxamine; PDI, protein disulfide isomerase; Fech, ferrochelatase; Ub, ubiquitin; RIPA, radioimmunoprecipitation assay; SA, succinylacetone; HMW, high molecular weight; ER, endoplasmic reticulum; K8 and K18, keratins 8 and 18.
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Associated with inherited porphyrias (3–5). Among the porphyrias, erythropoietic protoporphyria (EPP) and X-linked-dominant protoporphyria (XLP) result in the accumulation of PP-IX (4). Although EPP and XLP both accumulate PP-IX, the underlying etiologies of these two disorders are very different. In XLP, there is a gain-of-function mutation in ALA synthase 2 (erythroid-specific isoform of ALA synthase, ALAS2) leading to overproduction of ALA in bone marrow. For example, a C-terminal deletion in ALAS2 increased the total PP-IX by 27- and 24-fold in male and female patients, respectively (6, 7). Increased ALA modulates the flux of the heme biosynthetic pathway in a manner that insertion of Fe2+ into PP-IX by Fech becomes rate-limiting, thereby leading to PP-IX accumulation (6). On the other hand, PP-IX accumulation in EPP patients occurs due to an autosomal recessive, loss-of-function mutation in Fech (4, 8). Ferrochelatase activity decreases to almost 10–30% of normal, leading to PP-IX accumulation in erythrocytes, plasma, and liver, with levels of 3000–8000 nmol/g of tissue reported in EPP patients (9). Because Fech mutation is harbored in the bone marrow and liver (as the major heme factories), PP-IX accumulation occurs in both of these tissues. The excess PP-IX from bone marrow is transported in serum by albumin and then taken up by the liver (10). Thus, based on the source of PP-IX, protoporphyria could be classified as extrahepatic (e.g. XLP) or hepatic (e.g. EPP and XLP).

Protoporphyria (both EPP and XLP) is associated with several liver-related complications including cholelithiasis and parenchymal liver disease (in 5–20% of cases) that may be mild or progressive, potentially becoming end-stage (3, 4, 10). Recently, we demonstrated that the nuclear intermediate filament protein, lamin, aggregates in response to PP-IX-mediated liver damage (11). Profound aggregation of lamins A/C and B1 was observed in two different mouse models of protoporphyria and liver injury: (i) mice fed the porphyrinogenic drug 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) and (ii) mice that harbor a Fech mutation, which leads to PP-IX accumulation. In the current study we examined the cellular and biochemical consequences of extracellular exposure to PP-IX and intracellular accumulation of porphyrins (using ALA and the iron chelator deferoxamine (DFO)) as models to better understand intrahepatic and hepatic PP-IX-induced damage to cells. We also used mice fed DDC in parallel with the cell culture models and made special effort to separate ambient light from complete dark effects. Our results demonstrate subcellular compartment-selective injury, depending on the mode of porphyrin exposure. We show that several porphyrins cause selective protein aggregation, endoplasmic reticulum (ER) stress, and proteasome inactivation that are dependent on ambient light exposure. These alterations are likely to be mimicked in the hepatic and other porphyrias and may contribute to some of the disease symptoms.

Experimental Procedures

Cell Lines and Reagents—HepG2 and Hepa-1c1c7 cell lines were obtained from the American Type Tissue Culture Collection. HuH-7 cells (originally from the Japanese Collection of Research Bioresources Cell Bank) was a kind gift from Dr. Lei Yin (University of Michigan). HepG2 cells were propagated in Eagle’s minimum essential medium (Lonza, Walkersville, MD), Hepa-1c1c7 in minimum essential medium (Life Technologies), and HuH-7 in Dulbecco’s modification of Eagle’s medium (Cellgro, Manassas, VA). All three media were supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin, (Gibco). Animal studies were approved by the Animal Use and Care Committee at the University of Michigan. Male C57BL/6 mice (1 month old) were obtained from Taconic and were fed 0.1% DDC (Sigma-Aldrich) in LabDiet 5001 (PMI Nutrition International). Age- and gender-matched control mice were fed a standard mouse diet. Mice were euthanized by CO2 inhalation, and livers were harvested and snap-frozen.

Porphyrin-IX, deferoxamine mesylate, 5-aminolevulinic acid hydrochloride, succinylacetone, and N,N-dimethylacetamide were obtained from Sigma-Aldrich. Coproporphyrin III dihydrochloride and uroporphyrin III dihydrochloride were obtained from Frontier Scientific, Logan, UT.

Treatment of Cells with PP-IX or ALA+DFO—Cells were placed in 6-well plates and treated with 5 µM PP-IX in either complete (serum-containing) or serum-free medium for 1 h. After treatment, the cells were washed twice to remove residual PP-IX followed by cell harvesting by trypsinization. For the ALA+DFO treatment, cells were incubated in complete medium supplemented with 1 mM ALA and 100 µM DFO for 8 h, or otherwise as indicated. Cells were then harvested by trypsinization.

Preparation of Cell Lysates and Porphyrin, and Protein Extraction—The cell pellets, after washing, were solubilized in buffer containing 1% Nonidet P-40, 5 mM EDTA in PBS supplemented with protease inhibitors. The cell lysate was then pelleted to generate a “supernatant” fraction that includes the cytosolic and organellar compartments. The post-Nonidet P-40 pellet represents the nuclear fraction as well as remaining Nonidet P-40-insoluble components such intermediate filaments. The pellet was resuspended in a buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS (RIPA buffer) supplemented with protease inhibitor and then sonicated using 10–15 one-second pulses. Protein concentrations were measured using the BCA assay (Pierce BCA protein assay kit, Thermo Scientific). All incubations were carried out in the dark to minimize any potential photoactivation effects.

PP-IX was measured in the supernatant and the pellet fractions utilizing the intrinsic fluorescence of PP-IX. Briefly, 2 µl of the lysate was added to 200 µl of 1:1 mixture of ethanol: perchloric acid (0.9 N). The fluorescence of the resulting solution was measured in a Biotek Synergy HT 96-well plate reader using the filter sets 400/30 nm (excitation) and 590/35 nm (emission). The amount of PP-IX in the experimental samples was calculated by comparing the fluorescence value with a standard curve prepared using known PP-IX concentrations.

SDS-PAGE, Immunoblotting, and Mass Spectrometry—The supernatant (120 µg of protein) and pellet (20 µg of protein) fractions were analyzed by SDS-PAGE followed by immunoblotting and visualization by enhanced chemiluminescence. Antibodies were obtained from Santa Cruz Biotechnology Inc., Dallas, TX (lamin A/C, protein disulfide isomerase (PDI), and ubiquitin (Ub)); Abcam, Cambridge, MA (lamin B1); Cell Sig-
naling, Boston, MA (TSPO, CHOP, BiP, calnexin, IRE1α, and PERK); and Thermo Scientific (keratin 8 (clone TS1) and keratin 18 (clone DC10)). Mass spectrometry was carried out as described (11). The mass spectrometric data were sorted according to three criteria: (a) proteins with molecular mass less than 260 kDa, (b) proteins detected in both light-harvested and dark-harvested samples, and (c) >5-fold change in peptide-to-spectrum matches (as compared with untreated control).

Electron Microscopy and Nuclear Shape Morphometric Analysis—Transmission electron microscopy was carried out on cells fixed using 2% paraformaldehyde and 2% glutaraldehyde. Changes in nuclear shape were analyzed using a previously published method with minor modifications (12), using image analysis software ImageJ (13), available at http://imagej.nih.gov/ij/. Nuclear circularity was measured using the “circularity” plug-in of the “Measure” module in the software. The software defined circularity as \((4\pi \times \text{area})/\text{perimeter}^2\), and thus a perfect circle will have a circularity of 1 and any distortion will cause a decrease in circularity. The cut-off for misshapen nuclei was set at 0.65 (12), and we considered nuclei with <0.65 circularity as misshapen.

Proteasome Activity Assay—The 20S proteasome activity assay involved testing the cleavage of the labeled substrate peptide \(N\)-succinyl-LLVY-7-amido-4-methylcoumarin (Sigma-Aldrich), with release of the fluorescent amido-methyl-coumarin moiety and detection by the increase in fluorescence signal (14).

Cell lysates were prepared as highlighted above but without protease inhibitor. For the assay, 2 \(\mu l\) of the supernatant or pellet fractions was added to 88 \(\mu l\) of assay buffer (10 mM Tris-CI (pH 8.0), 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl). The reaction was initiated by adding 10 \(\mu l\) of the substrate peptide, to a final concentration of 40 ng/\(\mu l\). The change in fluorescence was continuously monitored using Biotek Synergy HT reader with the following filter sets, 360/40 nm (excitation) and 460/40 nm (emission) at 37 °C, for 90 min.

HPLC Analysis of Free Porphyrins—A Waters HPLC system, equipped with the Breeze software, with a binary pump solvent gradient and column heater (binary HPLC pump 1525, Waters), 717Plus Autosampler (Waters), and a fluorescence detector (2475 multi-wavelength fluorescence detector (Waters)) were used. A reverse-phase octadecyl silica (C18) Waters XSelect HSS T3 column (100-Å pore size, 3.5-\(\mu m\) particle size, 4.6 \(\times\) 150-mm dimension) HPLC column was used. The fluorescence detector was set at 402 nm (excitation) and 625 nm (emission) (15). The column (equilibrated for 10 min) was eluted at a flow rate of 1.0 ml/min with linear gradients of solvents A and B (solvent A, 0.05 mM monobasic sodium phosphate, pH 3.5, in water; solvent B, methanol). The solvent gradient was as follows: 0–4 min, 50–65% solvent B; 4–8 min, 65–85% solvent B; 8–22 min, 85–99% solvent B; 22–27 min, 99–10% solvent B; 27–30 min, 10–50% solvent B. After ALA/DFO or PP-IX treatments, the supernatant fractions were diluted 10-fold in HPLC injection solvent (50% methanol, 50% 0.05 mM monobasic sodium phosphate, pH 3.5), and 100 \(\mu l\) was injected. Retention times were determined using commercially obtained standards.

Treatment of HepG2 Cell Lysates with Porphyrin Intermediates—HepG2 cells were lysed to prepare the supernatant and pellet fractions. The protein concentration in each fraction (with the pellet being suspended and sonicated in RIPA buffer as described above) was adjusted to 2 mg/ml, followed by incubation with 50 or 150 \(\mu M\) PP-IX, uroporphyrin, or coproporphyrin for 30 min (22 °C) in a dark chamber. The reaction was quenched by adding reducing SDS-PAGE sample buffer, followed by gel separation and then immunoblotting.

Light and Dark Experiments—PP-IX powder was weighed in a microcentrifuge tube, and the solution was prepared under safe amber light illumination (filter number GBX2) in a dark room and was never exposed to ambient light. All dark experiments were carried out under these conditions. For example, experiments including the addition of PP-IX/ALA/DFO, incubation of cells, harvesting, preparing lysates, and centrifugation were all done in the dark room. For HepG2 cells, PP-IX or ALA/DFO was added to the medium and incubated in light or dark. Tissue culture plates were covered with foil and then moved to the tissue culture hood to completely protect from light exposure. After incubation, cells were harvested and lysed using RIPA buffer (under dark or ambient light conditions). An aliquot of the cell suspension was saved for protein and PP-IX quantification, while reducing SDS-PAGE sample buffer was added to the remaining fraction. SDS-PAGE analysis was also carried out in dark or ambient light conditions. For the mouse experiments, isolation and the entire experimental processing of the livers was also done either in ambient light or in the dark room. Livers were harvested and weighed and then snap-frozen in liquid nitrogen, wrapped in aluminum foil, and stored at −80 °C. The livers were homogenized (using a Dounce) in phosphate-buffered saline containing 1% Nonidet P-40, 5 mM EDTA, and protease inhibitor followed by pelleting. The pellet fraction was solubilized with RIPA buffer, followed by analysis of the lysates for PP-IX levels and by immunoblotting.

Solution Preparation—Protoporphyrin-IX was dissolved in N,N-dimethylacetamide (10 mg/ml stock solution). Uroporphyrin and coproporphyrin were dissolved in 1 \(\mu l\) NaOH to prepare 5 and 10 mg/ml stock solutions, respectively. Care was taken not to expose the stock solution to light. The stock solutions were diluted in phosphate-buffered saline (pH 7.4) immediately prior to use. ALA and DFO were prepared by dissolving the solids in water to yield 20 mg/ml stock solutions and then were filter-sterilized, aliquoted, and stored at −20 °C.

Results

Modeling Extrahepatic and Hepatic Protoporphyrin by PP-IX and ALA+DFO Treatment—We used two hepatocarcinoma cell lines, HepG2 (human) and Hepa (mouse), to develop an in vitro model to test the effect of PP-IX-mediated toxicity. Exogenous PP-IX was added in the medium to mimic extrahepatic porphyrinogenic stress as experienced by hepatocytes during XLP and EPP. Because serum is abundant in porphyrin-binding proteins (e.g. albumin), we examined the efficacy of serum-containing (complete medium) versus serum-free medium in delivering exogenous PP-IX to cells. PP-IX treatment in serum-free medium led to significantly higher PP-IX accumulation, as compared with complete medium, in the supernatant fractions.
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FIGURE 1. Induction of porphyrinogenic stress by exogenous PP-IX and ALA + DFO treatment. A, accumulation of PP-IX in the supernatant and pellet fractions after exogenous PP-IX or ALA + DFO treatment in HepG2 and Hepa cells. PP-IX and ALA + DFO treatment was done as described under “Experimental Procedures.” After the treatments, the Nonidet P-40 and pellet fractions were isolated followed by measurement of PP-IX content in triplicate samples. Statistical significance was determined using the unpaired t test (asterisks denote comparison with control, *, p < 0.05). Con, control. Error bars represent standard error of measurement. B, the supernatant and pellet fractions were subjected to SDS-PAGE and then Coomassie Blue staining to detect the HMW protein aggregates that did not enter the resolving gel, which can also be accompanied by a decrease in monomer detection. Left panel, dotted box versus closed box; PP-IX treatment in serum-free medium led to increased protein aggregates, compared to PP-IX treatment in complete medium. Right panel, closed box versus dotted box, the addition of SA to ALA + DFO led to a marked decrease in protein aggregation.

for both HepG2 and Hepa cells (Fig. 1A, top left panel). In the pellet fraction, a statistically significant increase was observed only in Hepa cells (Fig. 1A, bottom left panel). Next, we hypothesized that increasing the level of the porphyrin precursor ALA, with simultaneous blockade of ferrochelatase (by chelating free Fe²⁺ with DFO), will lead to intracellular accumulation of PP-IX and endogenous porphyrinogenic stress. Indeed, treatment of HepG2 and Hepa cells with ALA + DFO (1 mM and 100 µM, respectively, for 8 h) led to accumulation of PP-IX (Fig. 1A, right panel). PP-IX accumulation was completely abolished by co-treating the cells with succinylacetone (SA, 1 mM), a potent inhibitor of ALA dehydratase, which blocked ALA utilization, thus supporting the specificity of the treatment. Overall, intracellular PP-IX accumulation was significantly less (~5-fold) when cells were stimulated with ALA + DFO as compared with exogenous PP-IX. Additionally, after exogenous PP-IX treatment, the majority of PP-IX localized in the pellet fraction (~3× in the pellet versus supernatant fraction), but when stimulated with ALA + DFO, the majority of PP-IX was detected in the supernatant fraction (~3× in the supernatant versus pellet fraction). Because Hepa was less sensitive to ALA + DFO stimulation, HepG2 cells were used for the remaining set of experiments.

Importantly, in both HepG2 and Hepa cells, intracellular PP-IX accumulation was accompanied by protein aggregation based on the accumulation of high molecular weight (HMW) aggregates that are too large to enter the resolving gel. This is clearly evident after Coomassie Blue staining of the supernatant and the pellet fractions (Fig. 1B, arrowheads). The protein aggregation pattern (from a global perspective; but see Figs. 2–5 for specific protein alterations) paralleled the PP-IX accumulation trend. For example, in the supernatant fraction of Hepa cells, PP-IX treatment in serum-free medium led to increased aggregates, as compared with PP-IX treatment in complete medium (Fig. 1B, left panel, compare closed versus dotted box). Also as expected from the PP-IX measurement, the addition of SA to ALA + DFO led to a marked decrease in protein aggregation (Fig. 1B, right panel, compare dotted versus closed box).

Lamin Aggregation Parallels the Increase in PP-IX Accumulation—We tested lamin aggregation after exogenous PP-IX treatment and ALA + DFO stimulation, given our prior finding that lamins are a sensitive marker for PP-IX-induced liver damage because of their exquisite sensitivity to aggregate in association with porphyria-associated liver injury (11). Indeed, dramatic lamin B1 and A/C aggregation was detected in both Hepa and HepG2 cells (Fig. 2). HMW lamin aggregate formation correlates well with PP-IX levels. For example, lamin B1 aggregation (which can also be accompanied by a decrease in monomer detection) is higher in the absence versus presence of serum (Fig. 2A, compare lanes 6–8 with lanes 3–5; Fig. 2B, compare lanes 5 and 6 with lanes 3 and 4). As expected from the PP-IX measurement, HepG2 (but not Hepa) cells showed readily detectable lamin aggregation after treatment with ALA + DFO, which was abrogated when the cells were treated with ALA + DFO + SA (Fig. 2B, compare lanes 7 and 8 with lanes 9 and 10).

Because we observed dramatic nuclear lamin protein aggregation, we tested whether porphyrinogenic stress will cause changes in nuclear shape. For this, we used transmission electron microscopy to examine HepG2 cells treated with PP-IX or ALA + DFO. Representative examples of nuclei from each experimental group are shown in Fig. 3A–C. In control cells, the nuclei were round/circular in shape with no obvious deformity. In contrast, lobulation and invagination of the nuclear membrane were noted, particularly in the PP-IX-treated cells (Fig. 3D and E), thereby supporting the biochemical readout of lamin aggregation. Exogenous PP-IX treatment led to 34% of nuclei with <0.65 circularity, whereas untreated control and ALA + DFO had 11 and 13% of the nuclei below the cut-off, respectively. The limited effect on nuclear shape in the ALA + DFO-treated cells, as compared with PP-IX, is likely due to the much larger accumulation of PP-IX in the latter case (Fig. 1A: the porphyrins levels were 10,240 pmol/mg of protein (PP-IX treatment) versus 120 pmol/mg of protein (ALA + DFO treatment)). Thus, exposure to PP-IX leads to significant distortion of nuclear morphology in association with marked nuclear protein aggregation.
Cytoplasmic and Mitochondrial Proteins Show Different Susceptibility to PP-IX-mediated Aggregation—Profiling of the intracellular accumulation of porphyrin (Figs. 1 and 2) showed substantial PP-IX accumulation in the Nonidet P-40 fraction (which includes the cytosolic and solubilized organellar compartments) in addition to the Nonidet P-40-insoluble (pellet) fraction that includes nuclear and other extranuclear insoluble proteins. We examined potential protein aggregation in the cytoplasm of HepG2 cells, using the cytoplasmic intermediate filament protein markers keratins 8 and 18 (K8 and K18) (16) and the liver-specific fatty acid-binding protein 1 (FABP1) (17). Protoporphyrin-IX and ALA/DFO led to keratin aggregation in the pellet and supernatant fractions (Fig. 4A). However, there was no obvious aggregation or loss of monomer of the porphyrin-binding protein FABP1 (Fig. 4B), which indicates a level of selectivity for the observed PP-IX-mediated aggregation. Similarly, although PP-IX is synthesized in the mitochondria, there was no evidence of aggregation of the mitochondrial translocator protein TSPO (Fig. 4C), which is also known to bind to PP-IX (18). TSPO was tested in Hepa cells because it is not expressed in HepG2 cells (not shown).

PP-IX Accumulation Causes ER Protein Aggregation—Sustained perturbations of ER homeostasis have been implicated in several liver diseases (19). Hence we hypothesized that accumulation of PP-IX and associated protein aggregation will trigger an “unfolded protein response” leading to ER stress. We tested this hypothesis by examining biochemical changes in ER protein levels in the Nonidet P-40 supernatant fraction and determining whether there was evidence of ER protein aggregation (Fig. 5). Notably, BiP protein levels increased dramatically after both PP-IX and ALA/DFO treatment (Fig. 5A). The increase in BiP protein after PP-IX treatment was more than that observed in ALA/DFO-stimulated samples, in agreement with PP-IX accumulation levels under these conditions (2347 versus 534 pmol/mg of protein, respectively). However, for IRE1α and PERK, PP-IX treatment led to a significant loss in monomer with the presence of HMW cross-reactive species (Fig. 5A). On the other hand, ALA/DFO treatment caused no change in IRE1α and PERK monomers, but there were appreciably more HMW aggregates (Fig. 5A). In the case of CHOP, there was a complete loss of monomer after PP-IX treatment but no change after ALA/DFO stimulation (Fig. 5A). Similar formation of aggregates was noted for PDI and calnexin (Fig. 5B). Notably, despite lower PP-IX after ALA/DFO, aggregation of several ER proteins while maintaining similar monomer levels was clearly evident for PDI (Fig. 5B). We suspect that loss of antibody binding to the monomer is likely due to epitope masking and/or extensive aggregation. We did not observe any partitioning of

FIGURE 2. Lamin aggregation parallels PP-IX accumulation. A and B, Hepa (A) and HepG2 (B) cells were treated with PP-IX or ALA + DFO, followed by isolation of the pellet fractions. The pellet fractions (equal protein loading similar to that shown in Fig. 1B) were separated by SDS-PAGE and then immunoblotted with antibodies to lamin A/C and lamin B1. For each antibody, the lower panel shows the monomer after short chemiluminescence exposure (10 s), whereas the HMW aggregates are shown in the upper panel after a longer exposure (5 min). ND, not detectable.
the ER protein aggregates (or monomers) into the pellet fraction (not shown). Taken together, PP-IX accumulation leads to heterogeneity in the susceptibility of different ER stress proteins. This heterogeneity depends on whether the porphyrin source is exogenous (i.e. PP-IX exposure) versus endogenous (ALA + DFO), with the endogenous source rendering some ER proteins more aggregation-prone.

**Proteasome Function Is Impaired after PP-IX Accumulation**—Because our results showed that PP-IX accumulation causes prominent protein aggregation, we tested whether the ensuing proteotoxic changes have any effect on the proteasomal machinery. We hypothesized that proteasome activity will be compromised after PP-IX accumulation. In control cell Nonidet P-40 lysates, the addition of the proteasome substrate...
led to a linear increase of the fluorescence signal (after an initial lag phase) as a function of time as a measure of proteasome activity (Fig. 6A). By comparison, when cells were treated with PP-IX or ALA/H11001/DFO, there was a decrease in the slope and the amplitude of the signal, indicating proteasome inhibition. A similar pattern was observed for the pellet fraction as well, although the magnitude of the signal in the pellet fraction was 4-fold less than the supernatant fraction because the 20S proteasome is predominantly localized in the cytoplasmic fraction. Quantification of proteasome inhibition (as compared with untreated control) revealed that PP-IX and ALA/H11001/DFO inhibited the proteasome similarly (~30% and ~80% in the supernatant and pellet fractions, respectively, Fig. 6B). Given the proteasome inhibition, we hypothesized that there will be increased accumulation of ubiquitinated proteins. Indeed, when the HepG2 cell supernatant fraction was blotted for Ub, increased HMW Ub-containing aggregates were observed in PP-IX- and ALA/DFO-treated cells (Fig. 6C). Additionally, there was an increase in the mobility of the Ub monomer (PP-IX and ALA/DFO), and a decrease in monomer band intensity (ALA/DFO).

ALA/DFO Stimulation Generates Several Porphyrin Intermediates—As our results showed, there was an apparent mismatch between the extent of ER protein damage and the amount of accumulated PP-IX, particularly when the source of porphyrin is endogenous via ALA/DFO. For example, keratins are more susceptible to exogenous PP-IX (Fig. 4), whereas endogenous PP-IX accumulation via ALA/DFO preferentially affects ER proteins (such as PDI and calnexin) more prominently (Fig. 5). To explain this differential effect, we hypothesized that stimulating cells with ALA/DFO leads to formation of other upstream porphyrin biosynthetic porphyrin intermediates such as uroporphyrin and coproporphyrin, which may amplify the protein aggregation and toxicity. If so, then such porphyrin biosynthetic intermediates should accumulate and may even be released into the culture medium with continued ALA/DFO incubation times.

We tested this possibility by treating HepG2 cells with ALA/DFO for 2–24 h and then analyzing PP-IX levels fluorometrically in the cell supernatant and pellet fractions, as well as in the culture medium. As might be expected, there was a linear increase in total PP-IX accumulation as a function of ALA/DFO treatment time (Fig. 7A). PP-IX levels in the supernatant and pellet fractions increased initially and then saturated, whereas the levels in the medium were negligible in the initial 2–4 h, and then by 6 h, excess PP-IX was released into the medium. This suggests that there is an intracellular storage capacity for PP-IX that with time becomes overwhelmed with subsequent release of PP-IX. Next, we tested whether other unbound porphyrin intermediates were generated by ALA/DFO treatment. HPLC analysis revealed that PP-IX is the major porphyrin that is formed after ALA/DFO treatment,
cells (supernatant fraction) and the medium (Fig. 7, but uroporphyrin and coproporphyrin also accumulated in the cells (supernatant fraction) and the medium (Fig. 7, B and C). Analysis of free porphyrin intermediates being released in the medium after 24 h (Fig. 7D) highlighted clear differences in how cells handled these intermediates. For uroporphyrin, the retained and released amounts were nearly equal, whereas the

intracellular storage capacity for coproporphyrin and PP-IX is small with the major amount being released from the cell (Fig. 7D). In the case of treatment of cells with exogenous PP-IX, the major porphyrin species that accumulated was PP-IX (data not shown).

**Differential Effect of PP-IX, Uroporphyrin, and Coproporphyrin on Cellular Protein Aggregation**—To test the potential of uroporphyrin and coproporphyrin to mediate protein aggregation, we used a cell-free system comprising HepG2 cell lysate that was isolated and then challenged with two different concentrations (50 and 150 μM) of PP-IX, uroporphyrin, and coproporphyrin (Fig. 7, E and F). Notably, uroporphyrin was the most potent aggregation-causing agent for all three proteins that were tested, (PDI, K8, and lamin A/C), followed by coproporphyrin and then PP-IX. Thus, the differential effect that is observed after ALA +DFO treatment (Fig. 5) could be attributed, in part, to formation of these other porphyrin intermediates and subsequent amplified toxicity. Additionally, in this cell-free system, PP-IX-mediated protein aggregation was barely detected as compared with PP-IX-treated cells (e.g. Figs. 2 and 4). This is due to the low porphyrin to protein molar ratio in the cell-free system we used, and the relative insolubility of PP-IX in aqueous buffers as compared with uroporphyrin and coproporphyrin.

**Endogenous Porphyrin-mediated Toxicity and Protein Aggregation Occurs in Additional Cell Systems**—We have demonstrated the effect of exogenous PP-IX in human and mouse cell lines (HepG2 and Hepa). Because Hepa cells were not susceptible to ALA +DFO exposure (Figs. 1 and 2), we tested an additional human cell line, HuH7, which is also hepatic in origin. For this, HuH7 cells were treated with PP-IX or ALA +DFO, similar to HepG2 and Hepa cells. Concomitant to the level of porphyrin accumulation, and similar to what we noted in HepG2 cells (Figs. 2, 4, and 5), we observed PDI, lamin, and keratin aggregation, as well as up-regulation of BiP, coupled with proteasomal inhibition (Fig. 8). Similar findings were also noted in primary mouse hepatocyte cultures (not shown).

**Porphyrimediated Cellular Damage in Vivo and the Role of Ambient Light in Modulating the Toxicity**—To validate our in vitro findings, we used a pharmacologic porphyria model in mice. C57BL6 mice were fed 0.1% DDC, a hepatotoxin that leads to hepatic porphyrin accumulation (20). Given the photosensitivity of porphyrins, we tested the effect of light by harvesting and processing the liver samples, either under ambient laboratory light or in the dark room (see “Experimental Procedures”). As expected, both light-harvested and dark-harvested samples showed similar increases in the liver/body weight ratio, as well as in PP-IX accumulation (Fig. 9A). Next, we pooled the post-Nonidet P-40 pellet fractions from three livers for each group (control light-harvested and DDC light- and dark-harvested) and separated the proteins on SDS-PAGE in the dark followed by Coomassie Blue staining (Fig. 9B). Notably, both dark-harvested and light-harvested tissues showed material visible by Coomassie Blue stain at the top of the gel (Fig. 9B, marked by arrowhead in the inset). The upper parts of the gel (extending from the 250-kDa molecular mass marker to the bottom of the well, Fig. 9B) were excised, followed by mass spectrometry analysis to identify potentially aggregated pro-
teins. Fig. 9C shows a listing of the identified proteins that showed preferential enrichment as compared with the control sample (liver from non-DDC-treated mice). Notably, lamin aggregates were completely absent in the dark-harvested samples.

Next, we validated the mass spectrometric results by immunoblotting the pellet fractions with antibodies to K8/K18, lamin A/C, lamin B1 and GAPDH (Fig. 10). As reported earlier (16, 21), DDC feeding led to an increased expression of K8/K18 (which we observed in both light-harvested and dark-harvested livers, Fig. 10A). Dark-harvested livers had detectable K8/K18 aggregates, whereas the light-harvested livers harbored significantly more aggregates (Fig. 10A, upper panel, lanes 3–5 versus lanes 6–8; Fig. 10, B and C). This corroborated the mass spectrometry results, which suggested the presence of K8/K18 aggregates in light-harvested as well as dark-harvested samples. In contrast, for lamin A/C and B1, there were no detectable aggregates in the dark-harvested samples (Fig. 10A), which was also paralleled the mass spectrometry findings (Fig. 9C). However, there was a significant decrease in the lamin B1 monomer level in both light-harvested and dark-harvested samples (Fig. 10, A and B). We then tested GAPDH, which is known to form aggregates in the context of DDC-mediated liver damage (22). As shown in Fig. 9C, GAPDH showed comparable aggregates in light-harvested versus dark-harvested samples by mass spectrometry. Immunoblot analysis showed no difference in GAPDH monomer levels, but there was detectable aggregation in the light-harvested as well as dark-harvested samples (Fig. 10, A and C). Thus, protein aggregation is markedly attenuated in the absence of ambient light, but is still present in the dark-harvested liver from mice exposed to the porphyrinogenic drug for 5 days.

We also compared the light versus dark effect on protein aggregation in HepG2 cells because all the findings shown in Figs. 1–8 were carried out using ambient light during cell processing and gel electrophoresis. HepG2 cells were treated with PP-IX or ALA + DFO using light/dark conditions. Fig. 10D shows the results from the total cell extracts blotted with antibodies to the indicated antigens. A Coomassie Blue stain of each fraction is included to show equal protein loading.
earlier), which was absent when the samples were treated and processed in the dark (Fig. 10D, compare lane 3 with lane 2; lanes 6 and 7 with lanes 4 and 5). Experiments were also performed with HepG2 cell lysates (similar to conditions described for Fig. 7E) that were challenged in a cell-free system with PP-IX in dark and light conditions. As shown in Fig. 10E, lamin A/C aggregation was not detectable in the dark but was highly prominent when HepG2 lysates were exposed to PP-IX in the light (compare lanes 2 and 4). Thus, the relatively short-term/acute in vitro conditions do not favor formation of PP-IX-mediated aggregates in the dark. In contrast, in vivo subacute porphyrinogenic stress does result in detectable aggregates in the dark that become amplified in the presence of light.

Discussion

Protein Aggregation as a Mechanism of Cell Injury in Porphyria—Protoporphyria is associated with liver damage, and the extent of the liver damage depends on several factors including, but not limited to, the source of the porphyrin. For example, erythrocyte PP-IX levels in XLP patients were twice those in EPP patients, thereby predisposing these individuals to liver damage (8). Indeed, 17% of XLP patients (in a cohort of eight families with ALAS2 deletion) had overt liver disease, which is more frequent than the reported 5% frequency in patients with EPP (6, 9). XLP is extrahepatic, with its gain-of-function mutation in erythroid-specific ALAS2 leading to increased production of PP-IX in bone marrow (6, 8). In our study, mimicking “XLP-like” conditions by treating HepG2 and
Hepa cells with exogenous PP-IX led to 5–20-fold higher accumulation of PP-IX as compared with ALA/H11001/DFO treatment in both the supernatant and the pellet fractions (Fig. 1A). In addition, the majority of the PP-IX localized in the pellet fraction and was reflected by the increased lamin and keratin aggregation (Figs. 1–3). Our results suggest that exogenous PP-IX had an increased propensity to cause nuclear protein damage and thereby inflict hepatocyte and consequently liver damage. This was also reflected by the nuclear membrane deformations that were noted ultrastructurally (Fig. 3). In contrast, intracellular porphyrin accumulation via exposure to ALA/H11001/DFO leads to more prominent ER protein aggregation as compared with exposure to PP-IX (Fig. 5), whereas proteasome inhibition appears to be similar when comparing exogenous versus endogenously triggered accumulation (Fig. 6).

The liver plays a central role in heme biosynthesis, with 15% of heme biosynthesis occurring in liver (4). Liver is implicated as either the source or the target of excess porphyrins in porphyrias. For example, acute porphyria attacks are attributed to hepatic production of toxic porphyrin intermediates, which causes abdominal pain and neurologic symptoms including seizures (3), but how this toxicity occurs is unclear. On the other hand, EPP and XLP patients develop liver complications that may progress to end-stage liver failure (4). Our model (Fig. 11) and findings provide a potential mechanism for the cell and tissue injury that occurs in the context of different porphyrias. We posit that porphyria damage is attributed, in part, to protein aggregation that is caused by one or more porphyrin intermediates. The aggregation depends on the porphyrin and its availability, intracellular versus extracellular source, light exposure, and how efficiently porphyrins can be released extracellularly.

**The Dark Effect of Porphyrins**—Photodynamic activation of porphyrins and its use in cancer treatment are well known (23–25), but such use entails shining a laser to activate the porphyrins. In this regard, our results highlight the acute sensitivity of porphyrins in mediating protein aggregation given that our processing of the cell and tissue extracts in ambient light was enough to cause dramatic protein aggregation. Photosensitivity in porphyrias is indeed a major cause of morbidity. For example, six of the seven types of acute porphyrias are associated with photosensitivity (4). Our results indicate that porphyrin accumulation and exposure to ambient light are predicted to trigger and mediate a proteotoxic response. However, light exposure does not explain the porphyrin-mediated toxicity in internal organs such as liver. As we show in the experiments using mice, protein aggregation is markedly attenuated when the samples were completely shielded from ambient light. However, aggregate formation still took place in the dark (Figs. 9 and 10), albeit the levels were markedly less as compared with when the samples were processed in ambient light. Here the

**FIGURE 9.** Mass spectrometric analysis of liver tissues isolated using dark and ambient light conditions from mice with drug-induced hepatic porphyria. 1-month-old, male C57BL6 mice were fed DDC for 5 days, and the livers were harvested under ambient laboratory lighting or in the darkroom. A, liver/body weight ratio and total cellular PP-IX (normalized to protein content) in each experimental group (n = 4). The error bars represent standard error of measurement, and statistical significance was determined using the unpaired t test (asterisks denote comparison with control, *p < 0.05). B, the livers were homogenized in buffer containing 1% Nonidet P-40, and the insoluble pellet fraction was collected and solubilized by adding reducing SDS-PAGE Laemmli sample buffer. The pellet fraction from three different livers from each experimental group was pooled and separated by SDS-PAGE followed by Coomassie Blue staining to detect the HMW protein aggregates. The gel was excised along the dotted line followed by analysis of the HMW proteins by mass spectrometry. The inset shows the magnification of the portion of the gel that was excised showing the aggregates at the bottom of the well (arrowhead). C, the proteins that were identified from the excised gel are listed, with the fold-increase (for light- and dark-harvested tissues) after DDC feeding as compared with untreated control. Proteins marked by light shading (lamins, keratins and GAPDH) were validated by immune blotting as shown in Fig. 10.
aggregation phenotype in internal tissues (i.e. the liver) requires time, as was the case with DDC feeding. Interestingly, the protein aggregation signature seems to be different in light-exposed versus light-shielded samples. For example, as the current findings and earlier work (11) show, lamins are more susceptible to porphyrin-mediated protein aggregation as compared with keratins. In contrast, in the dark, keratins appear to be more susceptible than lamins (Figs. 9 and 10). However, this will require testing in different in vivo contexts, coupled with the finding that PP-IX effects (both light and dark) can be measured by aggregate formation and/or loss of monomer. Other studies have reported on the toxic potential of PP-IX in absence of light. For example, treatment of HepG2 cells with PP-IX led to a dose-dependent decrease in proliferation in absence of light (26). Additionally, there are reports of one-electron reduction of porphyrins to the respective anion-free radicals by hepatic microsomal enzymes (27, 28). Thus, in addition to the canonical pathway involving photoactivated reactive oxygen species generation, porphyrins might directly cause protein aggregation and cellular toxicity in the absence of light. Our results suggest that direct porphyrin damage via induction of protein aggregation is likely to be fundamental to the pathogenesis of the disease and its symptom complex, especially in the cases of cutaneous porphyrías and its associated photosensitivity (4). Of note, symptoms of porphyria remit and relapse (3, 5), so an attractive model is that symptom exacerbation is caused by an acute porphyrinogenic stress that results in protein aggregation and cell dysfunction. The exacerbation would then be cleared by refolding of the aggregates by induction of an unfolded protein response, normalization of the inhibition of the proteasome, or potential activation of autophagy to clear the aggregate. With regard to autophagy, the apoptosis that is induced by photoporation of the benzoporphyrin derivative, verteporfin, becomes enhanced in Hepa1 cells upon knockdown of the Atg7 (29). Verteporfin causes similar aggregation of the ubiquitin-binding protein p62 in cultured cells, (30), similar to what we previously observed in two porphyria experimental models (11).

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**FIGURE 10.** Dark versus light effect on porphyrin-mediated protein aggregation in tissues and in vitro. All isolations and SDS-PAGE analysis were done under ambient light or dark room conditions. A, livers harvested from DDC-fed mice were solubilized using Nonidet P-40 followed by separation of the insoluble pellet fraction by SDS-PAGE and then immunoblotting with antibodies to the indicated antigens. Monomer film exposure was for 10 s, whereas the exposure of HMW aggregates was for nearly 3 min. A Coomassie Blue stain of the pellet fraction is included to show equal protein loading. B and C, densitometric scanning of the films using ImageJ software to quantify the change in monomer and HMW aggregate levels after DDC feeding. Each data point is an average of readings from three independent livers, with the error bars representing standard error of measurement. Statistical significance was determined using the unpaired t test (*asterisks denote comparison with control, *, p < 0.05). D, immunoblot from HepG2 cell whole cell lysate, probed with antibodies to K8/K18. The cells were treated with 5 μM PP-IX for 24 h, in light (L) or dark (D) conditions. For ALA + DFO, the cells were treated with ALA (1 mM) and DFO (100 μM) for 8 or 24 h and then harvested in light or dark. PP-IX accumulation for each condition is included at the top of the lanes. A Coomassie Blue stain (CS) is shown for equal loading. E, a HepG2 cell free pellet fraction was treated with PP-IX (5 μM, 30 min) in ambient light (L) or in the dark (D) and then blotted with anti-lamin A/C antibody. Lanes 1–4 are from the same gel and had an identical exposure time (the two separate parts were spliced together along the dotted line).
Subcellular and Selective Proteotoxic Effects of Porphyrins—

Our findings demonstrate that porphyrins have pleotropic but also selective effects within subcellular compartments. This includes aggregation of the cytoplasmic cytoskeletal proteins K8 and K18, aggregation of the nuclear lamins, changes in nuclear membrane morphology, ER stress coupled with aggregation of several resident ER proteins, and inhibition of the proteasome. As we have demonstrated, PP-IX accumulation leads to ER protein damage and a unique form of ER stress by aggregating (and possibly inactivating) several ER stress-ameliorating proteins. Notably, for some ER proteins (PDI, calnexin), ALA/H11001/DFO caused more aggregation than PP-IX treatment, although the porphyrin accumulation was significantly less in ALA/H11001/DFO-treated cells (Fig. 5). We attribute this, at least in part, to the generation of other porphyrin intermediates such as coproporphyrin and uroporphyrin (Fig. 7), which amplified the toxic potential of ALA/H11001/DFO (Fig. 5).

The mechanism of PP-IX-mediated protein aggregation remains to be determined. Activation of transglutaminases by PP-IX is one possible mechanism that can result in covalent protein-protein cross-linking via isopeptide bond formation (11). However, inhibition of transglutaminases in cultured cells reversed lamin aggregation only partially (11). Direct formation of PP-IX (or other porphyrin) covalent adducts is also possible, but we have not been successful to date in showing direct evidence for the formation of such adducts (using mass spectrometry, not shown), and to our knowledge, no porphyrin-protein adducts have been well documented in mammalian systems to date. However, heme-protein covalent adducts via ester bond formation between the methyl groups of the porphyrin pyrrole rings and the carboxyl groups of acidic moieties of proteins such as lactoperoxidase are well established (31). Importantly, hemin does not cause any detectable protein aggregation (not shown), so the porphyrin moiety is the important ligand for aggregate formation. A more likely scenario, at least in part, is that porphyrins may bind to select proteins and then take advantage of their ability to generate noncovalent stacks to form a porphyrin-protein lattice (32). The protein aggregation effect of PP-IX is selective in that several proteins, including the known PP-IX-binding proteins FABP1 (17) and TSPO (18), are not prone to aggregation (Fig. 4). How some proteins are protected from aggregation remains to be investigated, but it is possible that some of these aggregation-resistant proteins may serve as genetic modifiers of liver injury. In summary, our findings provide a novel mechanism that may explain, in part, the cell and tissue damage that is associated with porphyrias.

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