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REVIEW

Porphyrin-Induced Protein Oxidation and Aggregation as a Mechanism of Porphyria-Associated Cell Injury

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SUMMARY

Porphyrias are caused by pathological accumulation of porphyrins and their precursors, with liver damage and cancer risk, photosensitivity, and neurovisceral involvement. Fluorescent porphyrins bind proteins reversibly and lead, in the presence of oxygen, to protein oxidation and aggregation with consequent cellular damage.

Genetic porphyrias comprise eight diseases caused by defects in the heme biosynthetic pathway that lead to accumulation of heme precursors. Consequences of porphyria include photosensitivity, liver damage and increased risk of hepatocellular carcinoma, and neurovisceral involvement, including seizures. Fluorescent porphyrins that include protoporphyrin-IX, uroporphyrin and coproporphyrin, are photo-reactive; they absorb light energy and are excited to high-energy singlet and triplet states. Decay of the porphyrin excited to ground state releases energy and generates singlet oxygen. Porphyrin-induced oxidative stress is thought to be the major mechanism of porphyrin-mediated tissue damage. Although this explains the acute photosensitivity in most porphyrias, light-induced porphyrin-mediated oxidative stress does not account for the effect of porphyrins on internal organs. Recent findings demonstrate the unique role of fluorescent porphyrins in causing subcellular compartment-selective protein aggregation. Porphyrin-mediated protein aggregation associates with nuclear deformation, cytoplasmic vacuole formation and endoplasmic reticulum dilation. Porphyrin-triggered proteotoxicity is compounded by inhibition of the proteasome due to aggregation of some of its subunits. The ensuing disruption in proteostasis also manifests in cell cycle arrest coupled with aggregation of cell proliferation-related proteins, including PCNA, cdk4 and cyclin B1. Porphyrins bind to native proteins and, in presence of light and oxygen, oxidize several amino acids, particularly methionine. Noncovalent interaction of oxidized proteins with porphyrins leads to formation of protein aggregates. In internal organs, particularly the liver, light-independent porphyrin-mediated protein aggregation occurs after secondary triggers of oxidative stress. Thus, porphyrin-induced protein aggregation provides a novel mechanism for external and internal tissue damage in porphyrias that involve fluorescent porphyrin accumulation. ***(Cell Mol Gastroenterol Hepatol 2019;8:535–548; https://doi.org/10.1016/j.jcmgh.2019.06.006)***

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Eukaryotic heme biosynthesis occurs in all eukaryotic cells with mitochondria,[1] and is an 8-step pathway spanning mitochondrial and cytoplasmic compartments (Figure 1*A*). The heme precursors, porphyrins, are cyclic tetrapyrroles with the pyrrole rings connected by carbon methene bridges and a central metal coordinating site. In mammalian systems, the metal center is occupied by Fe to form heme, Co (cobalt protoporphyrin-IX), or Zn (zinc protoporphyrin-IX). The pyrrole rings have 4 types of modifications (vinyl, methyl, ethanoate, propionate) in different combinations and positions depending on the specific porphyrin (Figure 1*A*). The modifications have profound effects on the physical properties of the

Abbreviations used in this paper: $^{1}O_2$, singlet oxygen; ABCB6, adenosine triphosphate-binding cassette sub-family B member 6 G2; ABCG2, adenosine triphosphate-binding cassette sub-family G member 2; ADP, ALA-dehydratase porphyria; AIP, acute intermittent porphyria; ALA, δ-aminolevulinic acid; ALAS, aminolevulinic acid synthase; BCRP, breast cancer resistance protein; CEP, congenital erythropoietic porphyria; CLPX, adenosine triphosphate-dependent Clp protease adenosine triphosphate-binding subunit clpX-like; Copro, coproporphyrin; CP, core particle; CPOX, coproporphyrinogen oxidase; Cyp3A1, cytochrome P450 3A1; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DFO, deferoxamine; EPP, erythropoietic protoporphyria; ER, endoplasmic reticulum; FECH, ferrochelatase; FLVCR1, feline leukemia virus subgroup C receptor-related protein 1; GOX, glucose oxidase; HCP, hereditary coproporphyria; IF, intermediate filament; NMP, N-methyl protoporphyrin-IX; PCT, porphyria cutanea tarda; PP-IX, protoporphyrin-IX; ROS, reactive oxygen species; RP, regulatory particle; Ub, ubiquitin; UPR, unfolded protein response; Uro, uroporphyrin; UROD, uroporphyrinogen decarboxylase; XLP, X-linked protoporphyria.

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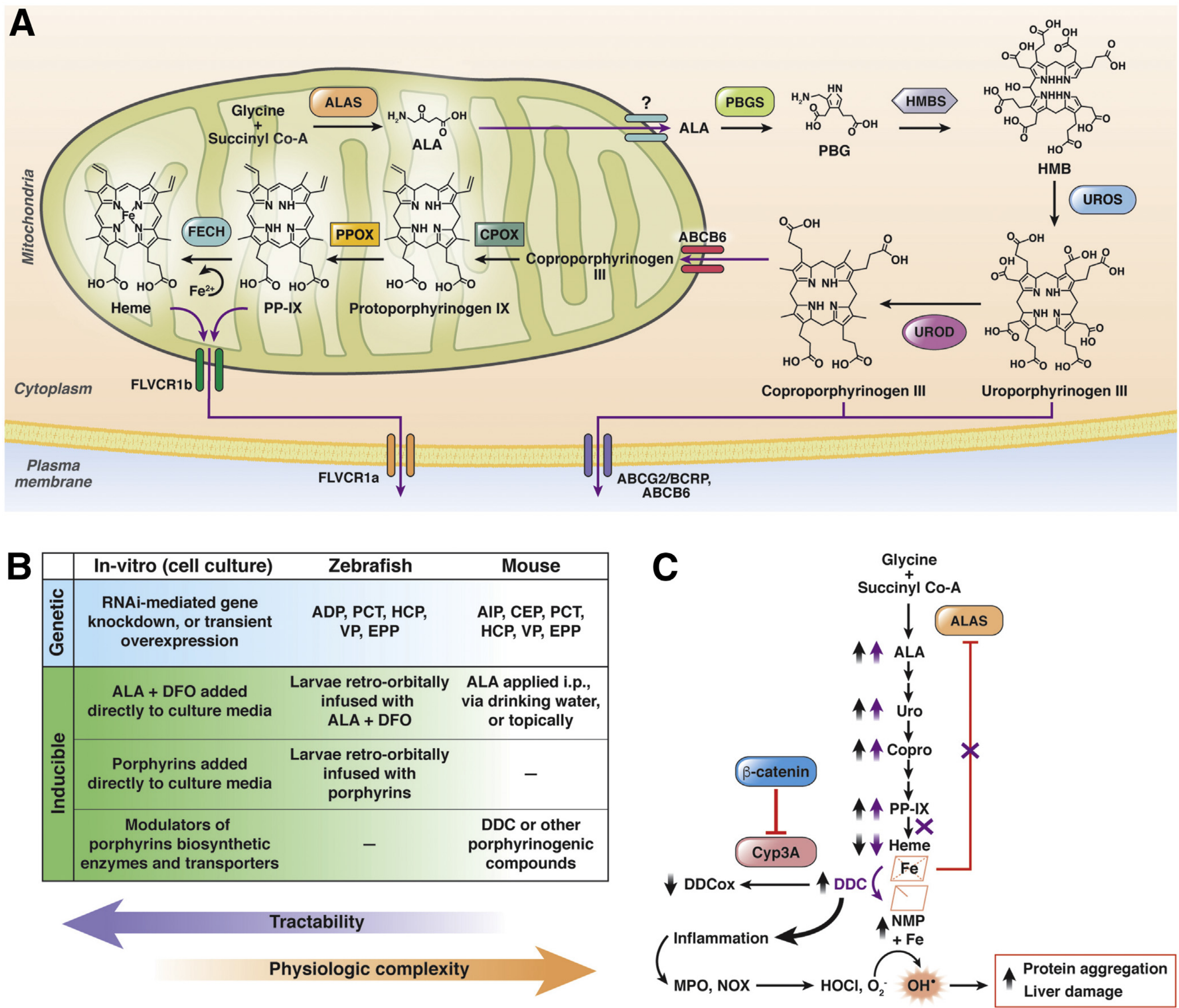

	In-vitro (cell culture)	Zebrafish	Mouse
Genetic	RNAi-mediated gene knockdown, or transient overexpression	ADP, PCT, HCP, VP, EPP	AIP, CEP, PCT, HCP, VP, EPP
Inducible	ALA + DFO added directly to culture media	Larvae retro-orbitally infused with ALA + DFO	ALA applied i.p., via drinking water, or topically
	Porphyrins added directly to culture media	Larvae retro-orbitally infused with porphyrins	–
	Modulators of porphyrins biosynthetic enzymes and transporters	–	DDC or other porphyrinogenic compounds

Figure 1. The heme biosynthetic pathway and experimental porphyria models. (*A*) Heme biosynthesis starts in mitochondria, where ALAS condenses glycine and succinyl Co-A to form the first committed metabolite of the pathway, ALA,[137] that exits mitochondria through an unidentified transporter/channel to the cytosol. In the cytosol, 2 molecules of ALA are cyclized by porphobilinogen synthase (PBGS) to form the monopyrrole, porphobilinogen (PBG).[55] Next, 4 molecules of PBG are joined by the hydroxymethylbilane synthase (HMBS) to form the linear tetrapyrrole, hydroxymethylbilane (HMB).[55] Ring closure of HMB by uroporphyrinogen synthase (UROS) forms the first cyclic tetrapyrrole of the pathway, uroporphyrinogen III, which is decarboxylated by UROD to form coproporphyrinogen III.[55] Coproporphyrinogen III enters the mitochondria through ABCB6,[97] where it is oxidized by coproporphyrinogen oxidase (CPOX) to protoporphyrinogen IX, which is further oxidized by protoporphyrinogen oxidase (PPOX) to form PP-IX.[55] In the last step of the pathway, FECH inserts Fe into the PP-IX molecule to form heme.[138] Heme and PP-IX can be exported from mitochondria through the transporter FLVCR1b,[139] and exported outside the cell by FLVCR1a[139–141] and ABCG2/BRCP.[96] In addition to heme and PP-IX, ABCG2/BRCP exports other cytosolic mono/tetrapyrroles including PBG, Uro, and Copro.[96] (*B*) Summary of the experimental models used to study porphyria. (*C*) Mechanism of DDC-mediated disruption of the hepatic heme biosynthesis that leads to porphyrin accumulation and protein aggregation in mouse liver. DDC methylates hepatocyte heme to form NMP (a potent ferrochelatase inhibitor). Methylation of the nitrogen atom of the pyrrole ring disrupts the Fe coordination and free Fe is released. Heme depletion, in turn, activates ALAS by the removing the feedback inhibitory effect of heme on ALAS.[142] Thus DDC through a combination of ferrochelatase inhibition (by NMP) and de-repressing ALAS (by decreasing heme levels), causes a buildup of porphyrins. In addition, hepatic inflammation-induced ROS including hypochlorous acid (HOCl) (from myeloperoxidase [MPO]) and superoxide (O^-_2) (from reduced nicotinamide adenine dinucleotide phosphate oxidase) react with free iron and generate (by the Fenton reaction) other potent oxidants, such as a hydroxyl radical (OH•),[143] which oxidize proteins and subsequently lead to protein-porphyrin aggregate formation. Through these combined actions, DDC feeding leads to hepatic porphyrin accumulation and liver damage. Hepatic Cyp3A1 metabolizes DDC to its inactive form DDC^{ox}, thereby diminishing the effect of DDC.[31] *β*-catenin modulates DDC-mediated porphyrin accumulation, protein aggregation and liver damage by blocking Cyp3A1, thereby increasing the porphyrinogenic potency of DDC.[75]

porphyrins, such as solubility and aggregation into macro structures.[2] Uroporphyrin (Uro)/coproporphyrin (Copro) (which contain 8 and 4 polar carboxylate side chains, respectively, Figure 1*A*) are hydrophilic, while apolar groups (eg, vinyl, methyl) and fewer carboxylates render heme/protoporphyrin-IX (PP-IX) hydrophobic. This in turn modulates porphyrins' biologic properties such as cell permeability and cellular retention (PP-IX>>Uro/Copro).[3]

Porphyrin and heme levels are maintained by several membrane transporters, including adenosine triphosphate-binding cassette sub-family B member 6 G2 (ABCB6), feline leukemia virus subgroup C receptor-related protein 1(FLVCR1a/b), and adenosine triphosphate-binding cassette sub-family G member 2 (ABCG2)/breast cancer resistance protein (BCRP) (Figure 1*A*). Heme is an essential co-factor for numerous enzymes and hemoproteins including hemoglobin, nitric oxide synthase, and cytochrome P450. In terms of tissue biosynthesis, 80% of heme is made in bone marrow, 15% in liver, with the remaining 5% coming from kidney and other tissues.[4] Given the cytotoxic nature of free heme and its precursors (Uro/Copro/PP-IX), heme biosynthesis is strictly regulated. Dysregulation of the heme biosynthetic pathway and subsequent abnormal accumulation of the heme precursors leads to the pathological condition, porphyria.

Porphyrias and Their Clinical Manifestations

Porphyrias, first reported by Schultz in 1874,[5,6] are a group of 8 disorders, each caused by a defect in a specific enzyme of the heme biosynthetic pathway, leading to accumulation of heme precursors (Table 1).[4] Of the 8 porphyrias, only X-linked protoporphyria (XLP) is caused by a gain-of-function mutation in ALAS2 (an erythroid-specific isoform of aminolevulinic acid synthase [ALAS]) thereby leading to overproduction of δ-aminolevulinic acid (ALA) and porphyrins in erythrocytes and bone marrow erythroid cells.[7] The remaining 7 porphyrias are caused by acquired or inherited loss of function of relevant enzymes that block pathway progression and lead to accumulation of the preceding substrates. For example, type-I porphyria cutanea tarda (PCT) is an acquired defect of hepatic uroporphyrinogen decarboxylase (UROD) activity (<25% of normal) (Figure 1*A*, Table 1).[8] Type-I PCT is acquired through excess alcohol, viral infection (particularly human immunodeficiency virus and hepatitis C virus), smoking, iron overload, and estrogen treatment.[8–10] In addition to the inherited genetic defects, multiple susceptibility factors can exacerbate the clinical manifestations of porphyria and trigger acute porphyric attacks.[11] For example, barbiturates and steroids (acute intermittent porphyria [AIP]),[12,13] and anabolic steroids (hereditary coproporphyria [HCP])[14] are contributing risk factors. The clinical features of porphyria are heterogeneous and their manifestations depend on the type of porphyria.[4] These features include neurovisceral manifestations (abdominal pain, constipation, seizures), photosensitivity, liver damage, and increased risk of hepatocellular cancer. Of these, photosensitivity (ranging from bullous/erosive photodermatosis to acute painful photosensitivity) is the most common feature that occurs in all porphyrias, except for AIP and ALA-dehydratase porphyria (ADP) (Table 1). Different degrees of liver damage are also a common feature of porphyrias including ADP, AIP, erythropoietic protoporphyria (EPP), HCP, PCT, and variegate porphyria (VP). The extent of liver damage varies, with a small subset of patients developing end-stage liver disease requiring liver transplantation.[15]

Experimental Models of Porphyria

Several model systems for studying porphyria have been generated (Figure 1*B*), as detailed subsequently. These models mimic the various etiologies of porphyria to different degrees and have unique benefits and limitations. Ultimately, the choice of the model system depends on the question being asked.

In Vitro Models

When cultured cells are treated with ALA and deferoxamine (DFO) (an iron chelator), there is accumulation of PP-IX, Copro, and Uro.[3,16] We have utilized this system to induce endogenous porphyrinogenic stress in primary mouse hepatocytes and various cell lines, including HepG2, Huh-7, Hepa-1c1c7, and n-TERT keratinocytes, and studied cellular and secreted porphyrin speciation.[3,16] Additionally, hydrophobic porphyrins, such as PP-IX, zinc protoporphyrin-IX, and PP-IX dimethyl ester, are taken up by cells when added to culture media.[3,16] The major advantage of using in vitro models is that they provide a reductionist approach to study the effect of porphyrins in different tissue cell types. For example, they could be utilized to assay putative porphyrin binding and transporting proteins and how they modulate cellular porphyrin uptake.[3,16] Also, RNA interference–mediated gene knockdown and/or transient overexpression coupled with porphyrin treatment could be utilized to study the role of specific enzymes and transporter proteins that may be involved in porphyria. In addition, cell culture systems provide useful models for testing small molecule modulators of porphyrin import, biosynthesis, export, and turnover. Although in vitro models offer several advantages, they lack the physiologic complexity of in vivo systems such as mouse and zebrafish models described subsequently.

Mouse Models

Numerous genetic mouse models of porphyria have been described in which a particular gene in the porphyrin biosynthetic pathway is deleted/mutated, causing a blockade of the pathway and accumulation of porphyrin precursors proximal to the alteration. These include models of AIP,[17] congenital erythropoietic porphyria (CEP),[18,19] PCT,[20] HCP,[21] VP,[22] and EPP (ie, all except XLP and ADP).[23,24] Many of these models phenocopy the pleiotropic features of human disease. For example, EPP mice are characterized by hepatic and erythrocyte porphyrin accumulation, liver damage, and acute photosensitivity.[4] The EPP mouse model harbors a ferrochelatase (FECH) mutation (which decreases hepatic FECH activity to 3% of wild-type) and mimics several features of EPP, including

Table 1. Classification and Clinical Features of the Genetic Porphyrias

Porphyria	Classification	Defective Enzyme	Enzyme Activity (% of Normal)	Inheritance Pattern	Increased Precursors	Clinical Manifestations
X-linked protoporphyria	Erythropoietic, cutaneous	ALAS2	>100	X-linked	PP-IX, Zn-PP	Photosensitivity, rapid onset of painful and itchy skin post–sun exposure, liver damage, end-stage liver disease
ALA-dehydratase porphyria	Hepatic, acute	ALAD	~5	AR	ALA, Copro III, Zn-PP	Neurovisceral (abdominal pain, peripheral neuropathy, seizures, behavioral changes), liver damage, end-stage liver disease
Acute intermittent porphyria	Hepatic, acute	HMBS	~50	AD	ALA, PBG	Neurovisceral, liver damage, end-stage liver disease
Congenital erythropoietic porphyria	Erythropoietic, cutaneous	UROS	1–5	AR	Uro I, Copro I	Severe photosensitivity, scars, hemolysis, erythrodontia, disfigurement of face and hands
Porphyria cutanea tarda	Hepatic, cutaneous	UROD	<20	Acquired (type I), AR (type II)	Uro I, Uro III, Hepta, Isocopro	Photosensitivity, skin fragility, slow onset of painless blisters, scars, increased risk for HCC
Hereditary coproporphyria	Hepatic, acute, cutaneous	CPOX	~50	AD	ALA, PBG, Copro III	Acute attacks, neurovisceral, photosensitivity, skin fragility, blisters, increased risk for HCC
Variegate porphyria	Hepatic, acute, cutaneous	PPOX	~50	AD	ALA, PBG, Copro III, PP-IX	Acute attacks, neurovisceral, photosensitivity, chronic liver abnormalities
Erythropoietic protoporphyria	Erythropoietic, cutaneous	FECH	20–30	AR	PP-IX	Photosensitivity, rapid onset of painful and itchy skin post–sun exposure, liver damage, end-stage liver disease

Increased precursors are found in body fluids (urine, blood), tissues, and stool.[4,9,10,15,149–157] AD, autosomal dominant; ALA, δ-aminolevulinic acid; ALAS, aminolevulinic acid synthase; AR, autosomal recessive; Copro, coproporphyrin; CPOX, coproporphyrinogen oxidase; FECH, ferrochelatase; HCC, hepatocellular carcinoma; Hepta, heptacarboxyl porphyrin; HMBS, hydroxymethylbilane synthase; Isocopro, isocoproporphyrin; PBG, porphobilinogen; PP-IX, protoporphyrin-IX; PPOX, protoporphyrinogen oxidase; Uro, uroporphyrin; UROS, uroporphyrinogen synthase; Zn-PP, zinc protoporphyrin-IX.

photosensitivity (eg, the animals require housing in opaque cages to avoid developing skin lesions).[23] In addition, inducible mouse models of porphyrias have been described. Similar to cell culture, many of these models involve administering ALA to mice, either topically,[25] intraperitoneally,[26] or through drinking water,[27] thereby resulting in porphyrin accumulation in several tissues. Similarly, mice fed ALA with iron and polychlorinated biphenyls develop PCT by selective inhibition of UROD.[20,28] Another strategy is to feed mice porphyrinogenic compounds such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), a heme methylating agent, which generates the potent FECH inhibitor N-methyl protoporphyrin-IX (NMP).[29,30] This leads to high levels of porphyrin accumulation in liver and erythrocytes, and causes significant liver damage (Figure 1*C*).[31] Similarly halogenated aromatic hydrocarbons, which are potent inhibitors of UROD, are used to induce porphyria in mice.[32,33] The advantage of using mouse models is that they offer relevant physiologic complexity and some similarity to humans.

Although mouse models are beneficial, limitations exist in terms of mimicking the pathologic nature of disease in patients. For example, several EPP models—m1Pas FECH mutant,[23,34] FECH exon 10 deletion[35,36]—provided valuable insights toward understanding the pathophysiology of EPP, but they do not fully mimic the commonly found (~97% of all FECH-related EPP cases) c.315-48C polymorphism.[24,37] Given the high incidence of the c.315-48C polymorphism, it is a common therapeutic target for EPP.[38,39] To address the unmet need for a suitable preclinical EPP model, a humanized mouse EPP model carrying c.315-48C polymorphism has been developed.[24] Though limitations still exist, mouse models are important for preclinical studies including hepatocyte transplantation[40] and gene transfer.[41–43]

Zebrafish Models

Zebrafish are a powerful model organism since they mimic the physiologic complexity of humans heme biosynthetic pathway but are more amenable to high throughput studies, and drug and genetic screening, than mouse models.[44–46] Fluorescent porphyrins (PP-IX/Uro/Copro) can be readily tracked and quantified in transparent zebrafish larvae with basic imaging techniques. Several genetic zebrafish models have been reported including *montalcino* (protoporphyrinogen oxidase deficiency),[47] *yquem* (UROD deficiency),[48] and *dracula* (FECH deficiency).[49] Genetic models of porphyria in zebrafish produce consistent

phenotypes in offspring including photosensitivity.[50] Targeted silencing with morpholinos and gene disruption with transcription activator-like effector nucleases or clustered regularly interspaced short palindromic repeats have also been used to generate porphyria models.[51,52,54] Inducible porphyria models in zebrafish have also been generated by retro-orbitally injecting porphyrins or ALA+DFO.[53] Injecting PP-IX or ALA+DFO caused accumulation of PP-IX in the liver (similar to EPP),[53] whereas injection of Uro I resulted in accumulation of Uro I in the bones and teeth of zebrafish, similar to CEP (unpublished observations). These zebrafish models mimic the various etiologies of porphyria to different degrees and have unique benefits and limitations. Ultimately the choice of the system depends on the question being asked, and typically a combination of different models offers advantage toward a better understanding of the disease.

Features of Porphyrin-Induced Protein Aggregation and Cell Damage

Porphyrin-Induced Oxidative Stress Causes Cell Damage

Given the site of synthesis, Uro and Copro localizes in the cytosol compared with PP-IX in mitochondria (Figure 1*A*).[4,55] However, several reports highlight the "ectopic" localization of porphyrins in other subcellular compartments, including the nucleus,[56] plasma membrane,[57] Golgi, endoplasmic reticulum (ER),[58] and lysosome.[59,60] Structural features of the porphyrins guide their cellular localization; anionic porphyrins (Uro, Copro, PP-IX, with their carboxylates) (Figure 1*A*) tend to localize in the acidic compartment of the lysosome.[59] Porphyrins also show preferential intracellular retention.[3] For example, upon ALA+DFO treatment of cultured cells, Uro and Copro are preferentially secreted into the culture media while PP-IX is mostly retained intracellularly.[3] Until recently, the paradigm has been that porphyrin-mediated cellular damage occurs by reactive oxygen species (ROS) generated through type I/II photosensitized reactions of porphyrins.[61–63] Several biological macromolecules (eg, lipids) act as electron rich nucleophiles to the electrophilic porphyrin-generated ROS. Indeed porphyrin accumulation has been reported to cause significant lipid peroxidation.[64] However, several clinical and recent experimental observations suggest that additional effects are likely to be involved. For example, porphyrin-photosensitized ROS generation is a viable explanation only for the photosensitivity, and does not explain how porphyrins damage internal organs. Also, specific targets of porphyrin-generated ROS oxidation were poorly understood, but recent work has demonstrated the unique property of porphyrins to cause organelle-selective protein oxidation and aggregation,[3,16,65] which we posit to be a major mechanism of cellular injury in porphyria. Notably, lysosomal porphyrins generate significantly less ROS such as singlet oxygen ($^1O_2$)[66] and do not appear to cause prominent protein aggregation,[3] possibly due to the inhibitory effect of the acidic pH on porphyrin-mediated protein damage.[16] The porphyrin effects on other organelles, in addition to the associated proteins that aggregate, are discussed subsequently.

Porphyrin Accumulation Leads to Intermediate Filament Protein Aggregation and Nuclear Shape Alterations

Intermediate filament (IF) proteins are 1 of the 3 major cytoskeletal protein families.[67,68] The major cytoplasmic IF proteins in epithelial cells are keratins (eg, K8/K18 in hepatocytes, K5/K14 in basal keratinocytes) and lamins A/C and B1 in the nucleus.[69] Keratins and lamins play important roles in cellular homeostasis and maintaining mechanical and structural integrity by acting as a scaffold, targeting of proteins to subcellular compartments, and modulating cell-signaling.[69] They are also implicated in gastrointestinal organ pathologies, including liver and pancreas.[67,70] Several studies utilizing genetic (FECH mutant)[65,71] and inducible (DDC-feeding)[31,65,71] models of porphyria demonstrated that PP-IX caused K8/K18 aggregation in association with Mallory-Denk body hepatocyte inclusion formation.[65] Aggregation was also observed in nuclear IF proteins, lamin A/C and B1, which occurs earlier than keratin aggregation.[65] Aggregation of IF proteins was also associated with morphologic changes in the cellular compartments. For example, porphyrin-mediated nuclear lamin aggregation caused nuclear ultrastructural deformation.[3,65] Aggregation of K8/K18 led to the formation of cytosolic electron-dense particles.[3,53,65] Several of these phenotypes, namely protein aggregation in the presence of light, were observed in vitro and with purified protein-porphyrin reaction mixtures,[3,16] indicating that this phenomena occurs due to direct protein-porphyrin interaction.

Porphyrin-Mediated ER Damage

The ER is the largest organelle in eukaryotic cells after the nucleus,[72,73] and is a site for protein quality control, folding, and posttranslational modifications.[74] Protoporphyrin-IX or ALA+DFO treatment of cells, or DDC-feeding to mice, caused prominent upregulation and/or aggregation of binding-immunoglobulin protein, an ER resident heat shock 70 kDa protein chaperone.[3,75] Although several other ER stress–related proteins also aggregated (eg, protein disulfide isomerase, calnexin, serine/threonine-specific protein kinase/endoribonuclease inositol-requiring enzyme 1 alpha),[3,75] there was a difference in the extent of aggregation depending on the porphyrin and its source. For example, some ER proteins (protein disulfide isomerase and calnexin) aggregated more after ALA+DFO treatment than after PP-IX treatment,[3] which maybe attributed to increased susceptibility of these proteins to Uro/Copro than PP-IX.[3] Damage to the ER compartment was not limited to protein aggregation since electron microscopy also revealed distortion of the ER after porphyrin accumulation.[53] The canonical ER stress response is initiated by the accumulation of aggregated/misfolded proteins, and upregulation of protective chaperones to mitigate the damage, and is termed the unfolded protein response.[74] Porphyrin-mediated aggregation and inactivation of ER

chaperones might abrogate the ability of ER to mediate the unfolded protein response and lead to a novel form of ER damage.

Porphyrins Disrupt Protein Turnover by Aggregating and Inhibiting Key Components of the Protein Degradation Machinery

Protein turnover is a key facet of a healthy cell, and is performed by 2 major pathways, proteasomal degradation and autophagy. Poly-ubiquitination targets proteins to the proteasome[76]; structurally, proteasomes consist of a core particle (CP) and 2 regulatory particles (RPs).[77,78] Poly-ubiquitin (Ub) proteins bind to the RPs, which then deubiquitinates them and transfers them to the CP, which has protease activity, for degradation.[77,78] Several studies have demonstrated an inhibitory effect of porphyrin on the proteasome catalytic activity.[3,71,79] Additionally, biochemical and proteomic analysis showed that PP-IX selectively aggregates several subunits of RPs, thus preventing poly-Ub proteins from binding to the proteasome[16] and manifesting as increased accumulation of poly-Ub proteins.[3,16,53,71] Therefore, porphyrins inhibit the proteasome by impeding protease activity of CP, and aggregating RP subunits, thereby preventing poly-Ub proteins from binding to RP.

Another vital pathway for protein turnover is autophagy, which involves p62/sequestosome 1–bound poly-Ub proteins that are targeted to the autophagosome where they undergo proteolytic degradation.[80] Porphyrins aggregate several components of the autophagic machinery including p62 and microtubule-associated proteins 1A/1B light chain 3B.[3,16,75,81,82] p62 is also known to aggregate upon autophagy induction,[83] but porphyrin-mediated p62 aggregation is likely to take place via direct porphyrin-protein interaction since it is also observed in PP-IX treated cell-free extracts.[75] Of note, Ub and p62 aggregates are present in Mallory-Denk bodies[84] in livers of DDC-fed mice.

Porphyrins Cause Cell Growth Arrest by Aggregating Proteins That Control Cell Cycle Progression

In addition to the protein aggregation and overall disruption of cellular protein homeostasis, porphyrins also cause cellular energy imbalance by aggregating key glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase,[3,85] and lead to mitochondrial dysfunction and decreased adenosine triphosphate synthesis.[86,87] These are predicted to have a profound effect on cell growth. In support of this, exposure of cells to ALA+DFO results in cell growth arrest in association with aggregation of several key cell cycle proteins including proliferating cell nuclear antigen, cyclin B1, and cyclin dependent kinase 4.[16]

Genetic Modifiers for Porphyrin-Mediated Damage

An outstanding question in the field of porphyria is why the causative porphyria mutations show such a wide range of penetrance in patients. For example, although EPP causes liver damage, only ~5% of patients develop end-stage liver disease that requires liver transplantation.[88] Similarly, for acute hepatic porphyrias (AIP/HC/VP), although there is a 50% reduction in enzyme activity, a wide range of pathogenicity is observed for different gene variants.[89] This raises the hypothesis that porphyria patients harbor genetic modifiers that increase or decrease the extent of heme biosynthesis disrupting mutations. If so, this in turn may contribute to the extent of porphyrin-triggered protein aggregation and its consequences. Some of the potential candidate genetic modifiers of porphyria end-organ damage are discussed subsequently.

Serum Porphyrin Transporters

Most porphyrin production occurs in the bone marrow and liver,[4] which may lead to organ damage at distant sites such as skin and abdomen.[4,9] Porphyrins are transported from the biosynthetic source to target tissues through blood by binding to porphyrin binding proteins such as serum albumin,[90,91] haptoglobin,[92] hemopexin,[93] and high/low-density lipoproteins.[94] The presence of albumin in culture media, protected from exogenous PP-IX induced protein aggregation. Further studies are needed to define the contribution of serum porphyrin-binding proteins to the heterogeneity of symptom presentation and end-organ damage in porphyria patients.

Cellular Porphyrin Binding Proteins and Porphyrin Transporters

Cellular porphyrin levels are also modulated by porphyrin transporters including FLVCR1a/b, BCRP/ABCG2, and ABCB6 (Figure 1*A*). Loss/gain-of-function mutations in these transporters might serve as genetic modifiers by sequestering porphyrins or increasing their uptake, and thereby exacerbating the symptoms. For example, Fukuda et al[95] demonstrated that ABCB6—mitochondrial Uro/Copro importer,[96,97] and cellular PP-IX exporter[98] (Figure 1*A*)—variants that are found in porphyria patients are nonfunctional. Indeed, deletion of ABCB6 in a FECH mutant mouse porphyria model increased porphyrin retention, and subsequent liver injury.[95] Of note, PP-IX is cleared from the body via bile but, not infrequently, high PP-IX concentrations in bile lead to canalicular plugs or development of pigment gall stones that may cause cholestasis and a feed-forward cycle impeding excretion of PP-IX and leading to further hepatic accumulation.[99] Thus, factors that promote cholestasis pose a risk for detrimental liver pathology in patients with EPP.

Another potential strategy for modulating intracellular porphyrin levels, that requires further investigation, is through cellular porphyrin-binding proteins, including glutathione S-transferase,[100] liver fatty acid binding protein,[101,102] heme binding protein 23,[103] SOUL/p22HBP proteins,[104,105] and glyceraldehyde 3-phosphate dehydrogenase.[106,107] These proteins could be protective or may induce porphyrin-mediated protein aggregation depending on their relative k_{on}/k_{off} of porphyrin binding vs that of the aggregating proteins such as keratins, lamins, and select ER proteins. Genetic variants of these porphyrin-binding

proteins could therefore contribute to the pathogenesis of some porphyrias.

Modulators of Porphyrin Biosynthesis

Indirect modulators of the porphyrin biosynthetic pathway such as adenosine triphosphate-dependent Clp protease adenosine triphosphate-binding subunit clpX-like (CLPX)[108,109] and β-catenin[75] may serve as porphyria genetic modifiers. CLPX is a mitochondrial unfoldase that interacts with ALAS to partially unfold it and enhance its enzymatic activity.[108] Notably, a dominant mutation in human CLPX, p.Gly298Asp, increases ALAS activity and PP-IX levels, and is found in patients with an EPP-like phenotype who do not harbor FECH mutations.[109] Another recently identified modulator of hepatic porphyrin biosynthesis is β-catenin.[75] β-catenin deletion significantly reduced mRNA levels of several heme biosynthetic enzymes, including ALAS, and protected from DDC-induced porphyrin accumulation, protein aggregation and liver damage.[75] The mechanism of DDC-associated porphyria occurs via DDC-mediated methylation of hepatic heme methylation to form NMP with release of free iron.[29,30] NMP then inhibits FECH, while heme depletion activates ALAS, which in combination lead to porphyrin accumulation. Porphyrins with the free iron, and other ROS/oxidants generated from the ensuing inflammation, create a toxic milieu that cause protein aggregation (Figure 1*C*). Notably, DDC is metabolized by cytochrome P450 3A1 (Cyp3A1) (a hepatic cytochrome P450) to its less toxic form, DDC^{ox},[31] and β-catenin downregulates Cyp3A1 expression and thereby increases DDC-mediated hepatic protein aggregation and damage.[75]

Mechanism of Porphyrin-Induced Protein Aggregation

PP-IX Causes Protein Aggregation Through Noncovalent Interactions With Proteins

One major difference of porphyrin-mediated protein aggregation from classical oxidative protein aggregation is that it occurs through noncovalent cross-linking.[16] Oxidative protein modification and subsequent aggregation typically occur through covalent bonding of oxidized amino acids Cys-Cys, Tyr-Tyr, transamidation (Lys-Gln), or carbonylation.[31,71,84,110–113] Porphyrins are capable of generating oxidants through either type I or type II photosensitized reactions (Figure 2). Both type I and II states are initiated when porphyrins absorb light to go to higher energy states (singlet/triplet), but type I photosensitization does not require oxygen.[61] Given that porphyrin-mediated protein aggregation requires oxygen,[16] an alternate pathway for protein aggregation is through type-II pathway generation of singlet oxygen, which oxidizes specific amino acids (eg, Met/Trp) in the protein.[16,114] Structural features of the porphyrin molecules are also important for protein aggregation. For example, esterification of the propionate group abolishes the ability of PP-IX to cause protein aggregation.[16] However, free heme, with its well-documented ability to generate dityrosine cross-linked protein aggregates[115–117] does not affect proteins that are susceptible to porphyrin-mediated aggregation. Fluorescent porphyrins (PP-IX, Copro, Uro), with de-protonated propionate groups may stack and bind to oxidized proteins through hydrophobic, electrostatic, and hydrogen-bonding mechanisms to form a lattice of porphyrin-protein aggregates (Figure 2).[16,118–120] Thus, uroporphyrinogen and coproporphyrinogen, which are nonfluorescent,[121] are predicted not to cause protein aggregation. Instead, unstable porphyrinogens undergo rapid auto-oxidation to the corresponding fluorescent porphyrins, Uro and Copro, which do cause protein aggregation.

Importantly, porphyrin-mediated protein oxidation does not appear to occur at random positions. For example, IF proteins, which have a central coiled-coil α-helical domain composed of heptad repeats represented as $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, where residues at position *a* and *d* are typically hydrophobic while residues *b, c* and in some cases *e, f, g* are charged,[122] become oxidized predominantly at positions *a* and *d*,[16] thereby predictably disrupting the heptad periodicity. In support of this model, a 30-residue peptide with heptad sequence repeats self-assembled to form millimeter scale fibrils after reacting with cobalt(III)-PP-IX.[123–125] Porphyrins could oxidize proteins with relative selectivity because of their ability to bind proteins in their native conformation, forming a sensitizer-acceptor complex.[16] Similar systems, where the oxidant source was coupled to its target, have also been reported.[126,127] Due to the noncovalent nature of the binding, porphyrin-protein complexes disaggregate when porphyrin is extracted.[16] We hypothesize that remissions of porphyria acute attack symptoms, where protein aggregation may be a contributing factor, might occur through this porphyrination/deporphyrination cycle (Figure 2).

How Do Porphyrins Cause Internal Organ Damage?

Porphyrin-mediated tissue damage is not limited to skin photosensitivity, but extends to internal organs, including liver damage in several porphyrias (Table 1). Indeed, qualitatively similar protein aggregation patterns were observed in livers of DDC-fed mice that were harvested and processed under safe-light illumination as compared with ambient light, although the extent of aggregation was markedly less in samples shielded from light.[3] As internal organs are not exposed to light, photosensitized porphyrin-protein aggregation is not applicable. To explain this discrepancy, we propose a 2-hit model. One hit is the porphyrin binding to select proteins in their native state that leads to localized unfolding (Figure 2). The second hit derives from internal organ oxidative stress that may derive from inflammation (macrophage infiltration and/or Kupffer cell activation) or other insults. This, in turn, leads to formation of ROS and protein oxidation.[128] Porphyrins then bind to the oxidized proteins further promoting aggregation (Figure 2). Support for this model was demonstrated in cultured hepatoma cells using glucose oxidase (GOX)–generated hydrogen peroxide as a secondary oxidant source. Cells treated with ALA+DFO in dark showed limited protein aggregation,[3,16] while

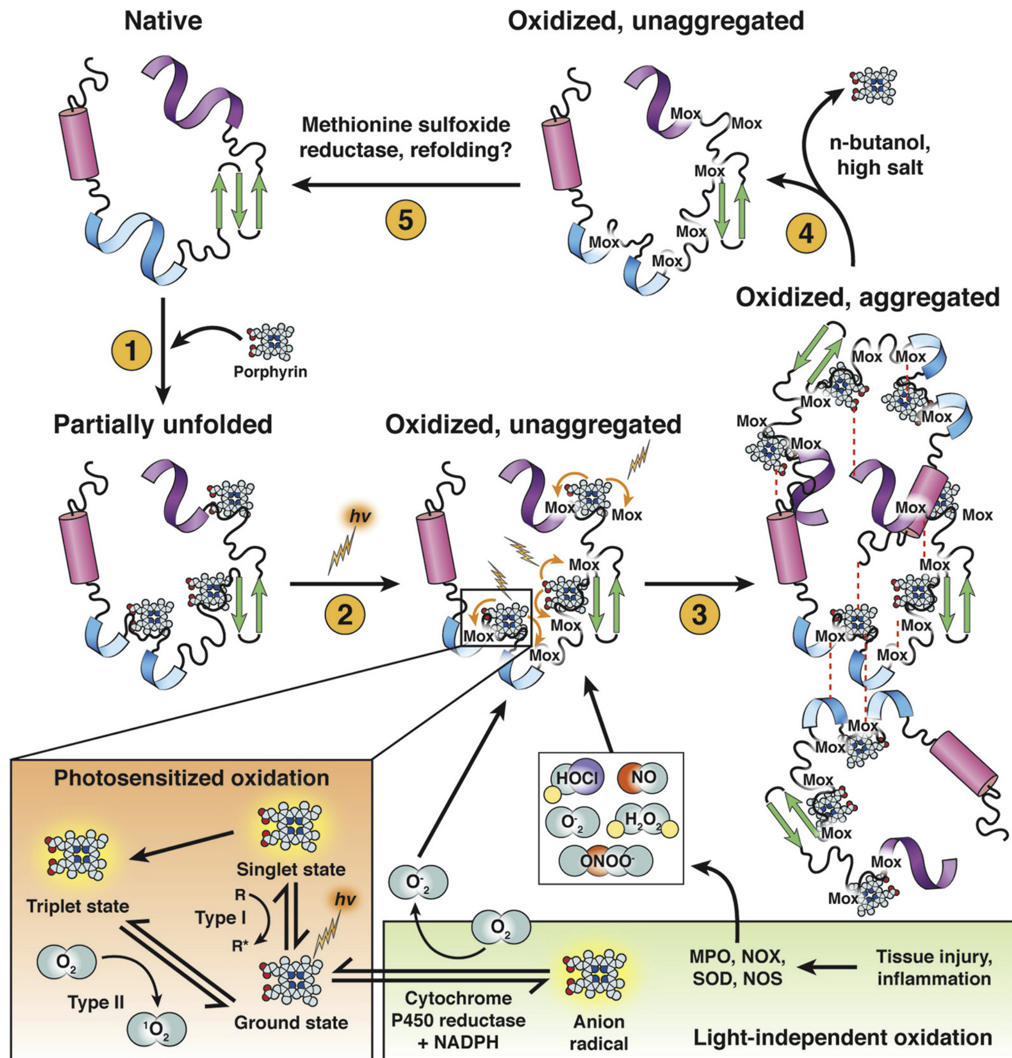

Figure 2. A porphyrination-deporphyrination cycle modulates porphyrin-induced protein aggregation in external and internal organs. The schematic shows the 5 steps of the reversible porphyrin-mediated protein aggregation. In step 1, PP-IX binds to proteins in their native states, independent of light,[16] leading to localized unfolding and conformational changes.[144,145] Porphyrin binding to target proteins helps circumvent the extreme labile nature of the ROS, namely $^1O_2$ (intracellular diffusion distance of 10–20 nm and a lifetime of 10–40 ns before it is quenched).[113,127,146] Photosensitized $^1O_2$ generation (see inset) by protein-bound porphyrins causes selective oxidation (step 2) and subsequently porphyrin-protein aggregates are formed through noncovalent interactions between oxidized proteins-porphyrins and porphyrin-porphyrin(s), (step 3). Clearance of porphyrin-protein aggregates may occur through deporphyrination (step 4), and reduction of the oxidized methionine residues through methionine sulfoxide reductase or chaperone assisted refolding (step 5), which remains to be tested. Alternatively, in internal organs and in the absence of light, oxidative injury such as inflammation, and hepatic cytochrome P450 reductase,[147,148] might act as an additional source of oxidants. For example, Uro is reduced by hepatic microsomal cytochrome P450 reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to an anion radical that decays back to the ground state thereby generating a O^{-}_2 radical.[147,148] These light-independent sources of secondary oxidants could generate oxidized protein-porphyrin aggregates. H_2O_2, hydrogen peroxide; Mox, methionine sulfoxide/sulfone; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase.

co-treatment with ALA+DFO and GOX led to readily detectable protein aggregate formation in the dark.[16]

Clinical Significance of Porphyrin-Mediated Protein Aggregation

Several facets of porphyric cell and tissue damage, especially in liver, can be accounted for by porphyrin-mediated protein aggregation. For example, lamin B1 aggregates have been described in liver explants from patients with alcoholic cirrhosis, similar to the effect of PP-IX–induced lamin aggregation.[65] Alcohol is a risk factor and trigger in acute porphyrias[11] and leads to inhibition of several heme biosynthetic enzymes, as well as to upregulation of hepatic ALAS-1.[129] Acquired PCT, associates with excess alcohol consumption, hepatitis C, excess iron[8] that, in turn, provide a pro-oxidant toxic milieu. Porphyrin accumulation in absence of light causes protein aggregation when a secondary source of oxidant

such as GOX is present,[16] though additional clinically-relevant sources of oxidants remain to be tested. Thus, we posit that fluorescent porphyrin accumulation, combined with oxidative stress, act in a feed forward manner to form porphyrin-oxidized protein aggregates. On the other hand, for acute porphyrias, ALA/porphobilinogen (PBG) levels increase (Table 1) in association with neurovisceral symptoms. Although, protein aggregation in neural tissues has not been tested, it is possible that the increased ALA/PBG (which cross the blood-brain barrier)[130–132] may enter neural cells and induce porphyrin accumulation to levels that may lead to protein aggregation. Given the reversible nature of porphyrin-protein aggregates,[16] via clearance mechanisms that remain to be defined, their transient accumulation could contribute to acute porphyria attacks. This speculation may find support by the observation that patients with EPP/XLP and with severe hepatopathy sometimes develop neurological features that resemble those of acute porphyria attacks.[133]

Future Directions

Porphyrias are now well understood in terms of their underlying genetic defects but remain difficult-to-treat disorders. Recent appreciation for the extent, selectivity and mechanism of porphyrin-mediated protein oxidation and aggregation hints at several potential novel therapeutic strategies that may be targeted. The mechanism of turnover of protein aggregates remains to be determined, and relevant to this is the potential use of proteasome inhibition[134,135] and chemical chaperones[136] in the treatment of CEP. Devising tools for biochemical in situ detection of porphyrin-generated protein aggregates may be possible by the generation of epitope-specific antibodies that recognize oxidized and neo-epitopes. Also, characterization of the aggregates using cryoelectron microscopy and other structural-biology tools is likely to be beneficial. Other key areas that remain to be understood include defining molecular signatures in proteins susceptible to porphyrin-mediated aggregation, whether such signatures are unique to different porphyrins, and understanding the genetic modifiers that modulate disease severity in patients with porphyria.

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