Biochimica et Biophysica Acta 1822 (2012) 1453–1464

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Primary hyperoxalurias: Disorders of glyoxylate detoxification ☆,☆☆

Eduardo Salido [a,*], Angel L. Pey [b], Rosa Rodriguez [a], Victor Lorenzo [a]

[a] Hospital Universitario de Canarias, Universidad La Laguna, and Centre for Biomedical Research on Rare Diseases (CIBERER), Tenerife 38320, Spain
[b] Departamento de Química-Física, Facultad de Ciencias, Universidad de Granada, 18071, Granada, Spain

ARTICLE INFO

Article history:
Received 2 December 2011
Received in revised form 17 February 2012
Accepted 6 March 2012
Available online 14 March 2012

Keywords:
Primary hyperoxaluria
Oxalate
Glyoxylate
AGXT
GRHPR
HOGA1

ABSTRACT

Glyoxylate detoxification is an important function of human peroxisomes. Glyoxylate is a highly reactive molecule, generated in the intermediary metabolism of glycine, hydroxyproline and glycolate mainly. Glyoxylate accumulation in the cytosol is readily transformed by lactate dehydrogenase into oxalate, a dicarboxylic acid that cannot be metabolized by mammals and forms tissue-damaging calcium oxalate crystals. Alanine-glyoxylate aminotransferase, a peroxisomal enzyme in humans, converts glyoxylate into glycine, playing a central role in glyoxylate detoxification. Cytosolic and mitochondrial glyoxylate reductase also contributes to limit oxalate production from glyoxylate. Mitochondrial hydroxyoxoglutarate aldolase is an important enzyme of hydroxyproline metabolism. Genetic defect of any of these enzymes of glyoxylate metabolism results in primary hyperoxalurias, severe human diseases in which toxic levels of oxalate are produced by the liver, resulting in progressive renal damage. Significant advances in the pathophysiology of primary hyperoxalurias have led to better diagnosis and treatment of these patients, but current treatment relies mainly on organ transplantation. It is reasonable to expect that recent advances in the understanding of the molecular mechanisms of disease will result into better targeted therapeutic options in the future. This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of peroxisomes in Health and Disease.

1. Introduction

Glyoxylate is a two-carbon (C2) keto-acid generated by intermediary metabolism, with glycine, hydroxyproline and glycolate as the best known sources in humans. Glyoxylate is a highly reactive aldehyde that is readily catalyzed by various dehydrogenases and oxidases, including lactate dehydrogenase (LDH), which converts it into oxalate, an end product of metabolism in mammals that has to be eliminated with the urine and tends to precipitate as tissue-damaging calcium oxalate. Since LDH is quite abundant in the hepatocyte cytosol and vertebrates lack the ability to use C2 compounds to produce TCA cycle intermediates via the glyoxylate shunt, most of the glyoxylate generated must be metabolized within organelles such as the peroxisome and mitochondria in order to limit oxalate production. To further control the levels of oxalate produced, cytosolic glyoxylate reductase (GRHPR) competes with LDH for glyoxylate, reducing it to glycolate, a highly soluble C2 molecule (Fig. 1).

In human hepatocytes, abundant alanine-glyoxylate aminotransferase (AGT) makes the peroxisome [1] an efficient 'glyoxylate sink', converting glyoxylate in glycine. Glyoxylate can be imported from the cytosol/mitochondria and it can also be produced in the peroxisome by either D-amino acid oxidase (DAO) or hydroxyacid oxidase (HAO1) (glycolate oxidase), using glycine or glycolate as substrate, respectively. The peroxisome membrane is permeable to glycolate, glyoxylate and other small hydrophilic solutes, largely due to the presence of the channel-forming protein PXMP2 [2]. This, combined with the fact that AGT can tolerate high glyoxylate concentrations without showing inhibitory action on the forward reaction [3] make the peroxisome a glyoxylate detoxifying compartment that shields the surrounding cytoplasm from glyoxylate accumulation and secondary oxalate production.

Mitochondria also play an important role in glyoxylate metabolism [4,5], mainly due to their capacity to metabolize hydroxyproline, through a series of four enzymatic reactions [6]. Collagen is a major constituent of extracellular matrix. Posttranslational modification of collagen by proline hydroxylase during the biosynthesis and maturation of collagen contribute to the stability of the collagen triple helix. Upon collagen turnover, the 4-hydroxyproline released cannot be reutilized and is degraded. The first two reactions of the pathway are analogous to those of proline degradation, but the first enzyme of

☆ Standardized nomenclature is AGXT to refer to the protein encoded by the *AGXT* gene. However, we will use AGT in this review to refer to the protein encoded by the gene *AGXT* because this is the term traditionally used.
☆☆ This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of peroxisomes in Health and Disease.

* Corresponding author. Tel.: +34 922319338; fax: +34 922319412.
E-mail address: esalido@ull.es (E. Salido).

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doi:10.1016/j.bbadis.2012.03.004

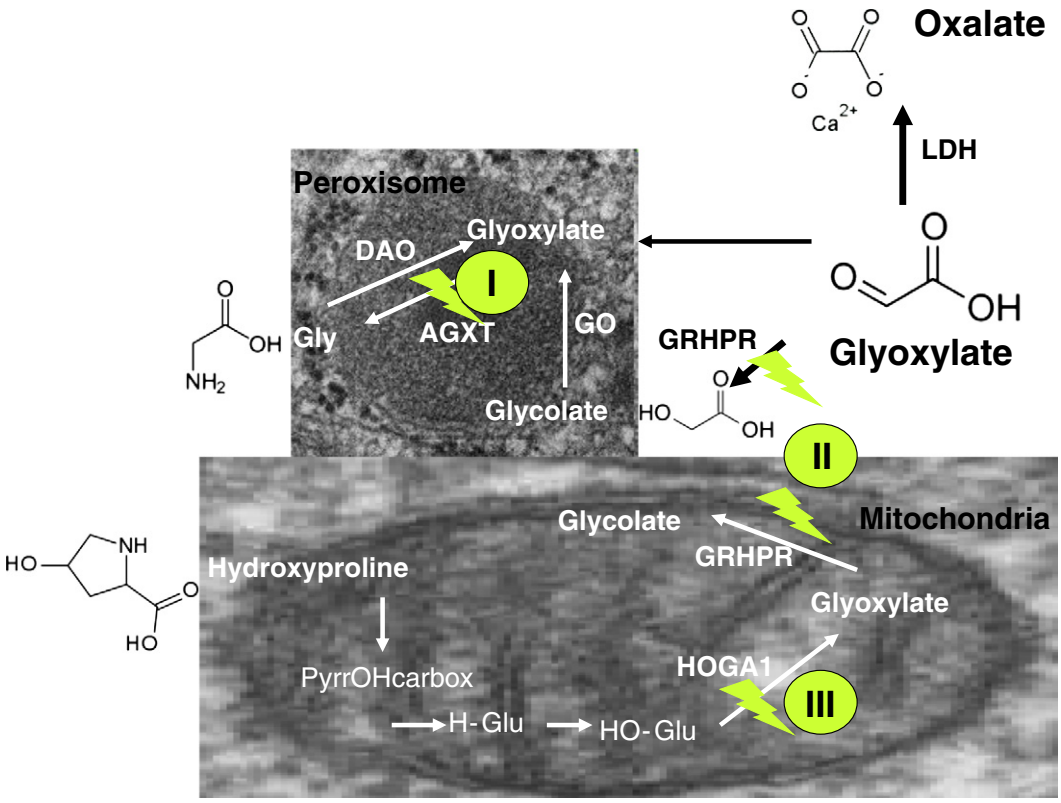

Fig. 1. Glyoxylate detoxification pathway in human hepatocytes. Simplified pathways involving glycine, glycolate and hydroxyproline as the main sources of glyoxylate. Peroxisomal glyoxylate is detoxified by AGT, while mitochondrial and cytosolic glyoxylate is reduced to glycolate by GRHPR, preventing excessive oxidation to oxalate by LDH. Hydroxyproline metabolism results in the production of 4-hydroxy-2-oxoglutarate that is normally split into glyoxylate and pyruvate by HOGA1. PyrrOHcarbox = pyrroline-5-carboxylate; H-Glu = 4-hydroxy-glutamate; HO-Glu = 4-hydroxy-2-oxoglutarate.

hydroxyproline degradation, hydroxyproline oxidase, is distinct from proline oxidase. Indeed, patients deficient in proline oxidase do not show elevated 4-hydroxyproline levels, while free hydroxyproline, but not proline, accumulates in the plasma and urine of individuals with hydroxyproline oxidase deficiency. The last step of the pathway is also specific to hydroxyproline degradation and involves the cleavage of 4-hydroxy-2-oxoglutarate (2-keto-4-hydroxyglutarate) into glyoxylate and pyruvate by 4-hydroxy-2-oxoglutarate aldolase (HOGA1). The glyoxylate can then be converted to glycolate by GRHPR. Since collagen contains ~15% hydroxyproline, its turnover results in an estimated release of 300–450 mg hydroxyproline a day, accounting for the daily production of 180–240 mg glyoxylate [7,8].

The glyoxylate detoxification pathway reflects the evolutionary origins of metabolic compartmentalization [9], and the subcellular distribution of its key enzymes has been under evolutionary pressure. Diet must have been an important component, since glycolate is abundant in vegetables while hydroxyproline is abundant in meat.

The subcellular distribution of AGT has been thoroughly analyzed in numerous mammals, and it has been shown to vary between peroxisomes and mitochondria in different species [10]. Significant correlation between AGT distribution and diet was found [11]: mitochondrial in carnivores, peroxisomal in herbivores, and both mitochondrial and peroxisomal in omnivores, suggesting that the variable subcellular localization of AGT results from dietary selection pressure.

The relevance of glyoxylate detoxification to human health is exemplified by the devastating consequences of inherited mutations in genes coding for key enzymes in this pathway (Fig. 1), resulting in mendelian diseases characterized by high oxalate content in the urine: primary hyperoxalurias (PHs).

2. Primary hyperoxalurias

PHs are rare inborn errors of glyoxylate metabolism that result in high oxalate production mainly by the liver [12–14]. Oxalate does not seem toxic to hepatocytes, but since it cannot be metabolized in mammals, it can only be filtered at the glomerulus and also secreted by the renal tubules (and to some extent the bowel also), with urinary excretion levels > 0.5 mmol/1.73 m^2 per day (typically > 1 mmol/1.73 m^2) in PH patients (normal oxalate excretion < 0.45 mmol/1.73 m^2 per day). The kidneys undergo progressive deterioration due to calcium oxalate deposition: urolithiasis, nephrocalcinosis and end stage renal disease (ESRD). The parenchymal deposition of oxalate (nephrocalcinosis) induces interstitial inflammation and fibrosis that result in progressive loss of renal function. The endogenous overproduction of oxalate, with normal dietary oxalate absorption [15], sets these mendelian disorders apart from secondary hyperoxalurias, which result from increased oxalate absorption in the gut. Secondary hyperoxalurias tend to be milder (<0.8 mmol/1.73 m^2 per day) and are usually a consequence of gastrointestinal diseases frequently associated to fat malabsorption: inflammatory bowel diseases, cystic fibrosis, bariatric surgery or short bowel syndrome [16]. However, severe cases of secondary hyperoxaluria can also lead to ESRD and systemic oxalosis.

The three genetic defects currently known to cause PH are inherited with autosomal recessive pattern. The genes involved are alanine-glyoxylate aminotransferase (*AGXT*, at 2q37.3, MIM*604285), for PH type I (PH1, MIM#259900), glyoxylate reductase/hydroxypyruvate reductase (*GRHPR*, at 9q12, MIM*604296), for PH type II (PH2, MIM#260000) and 4-hydroxy-2-oxoglutarate aldolase (*HOGA1*, also known as *DHDPSL*, at 10q24.2, MIM*613597), for PH type III (PH3, MIM#613616). Most of the PH families can

be mapped to one of these three loci, but there are a few patients with primary hyperoxaluria for which the genetic defect has not been found yet.

Around 80% of PH patients suffer PH1, a peroxisomal disease and the most severe PH type, usually resulting in ESRD at some point, although with a wide range of severity. At ESRD, the build-up of oxalate in the body (known as oxalosis) quickly results in bone, heart, skin and retinal complications and, unless liver-kidney transplantation is performed, death in most patients [17–19]. Given its statistical predominance, most epidemiological studies on PH, essentially refer to PH1.

PH has an estimated prevalence ranging from 1 to 3 per million population and an estimated incidence rate of ~1:100,000 live births per year in Europe [20–22], although the exact prevalence is difficult to assess, due to suspected underdiagnosis, with symptoms attributed to common kidney stone disease. The most comprehensive attempts to estimate the true incidence of the disease [22] have resulted in higher incidence rates than previously reported. Higher rates have also been found in historically isolated populations, like the Canary Islands, due to founder effect [19]. Although PH accounts for a little less than 1% of pediatric ESRD population in registries from developed countries [23], the disease is more prevalent in countries where consanguineous marriages are common. Approximately 10% of Kuwaiti children and 13% of Tunisian children with ESRD have been reported to suffer PH [24,25].

2.1. Primary hyperoxaluria type I

PH1 is caused by deficient or mistargeted AGT [26], a liver-specific enzyme that, in humans, is normally located in the peroxisomes only. From the early descriptions of PH1, it was reported that the majority of patients had glycolic aciduria and hyperoxaluria [27]. AGT catalyzes the transamination between L-alanine and glyoxylate, to produce pyruvate and glycine in the presence of pyridoxal 5′-phosphate (PLP) as cofactor, a reaction largely shifted to the formation of pyruvate and glycine [3].

AGXT cDNA was independently cloned by two groups [28,29], using probes from the orthologous rat gene [30]. The gene has 11 exons and spans ~10 kb [31], resulting in a 1.7 kb mRNA with a coding sequence of 1176 bp. The gene product AGT is a homodimeric protein, each 43 kDa subunit containing 392 amino acids and holding one molecule of PLP as cofactor [32]. The main N-terminal domain contains most of the catalytic active site, the cofactor-binding site, and the dimerization interface. The smaller C-terminal domain is known to interact with the peroxisomal receptor PEX5, targeting the dimer to the peroxisome. AGT, carrying a non-canonical peroxisomal targeting sequence (PTS1), is among the peroxisomal proteins with the weakest affinities for PEX5 [33]. An ancillary sequence surrounding amino acids 324–345 has been proposed to help the peroxisomal targeting of AGT [34]. A recently released crystal structure of the AGT in complex with the PTS1-binding domain of PEX5 (PDB: 3IMZ) also confirmed that residues 303–306 and 327–330 are largely buried upon binding. In fact, AGT binds to PEX5 with ~10-fold higher affinity than its PTS1 octapeptide, showing the functional role of this ancillary sequence in PEX5 binding (unpublished).

The 3-D structure of AGT (PDB: 1H0C) [32] has provided important information to better understand the function of the protein and the effect of changes in amino acid that account for a majority of the PH1 mutations. Important contributions to the physicochemical and enzymatic properties of recombinant AGT and some of its common variants have also been reported recently [35].

More than 150 mutations have been described for *AGXT*, which is expressed only in the liver, and they have been summarized recently [36]. Missense mutations are common, followed by small insertion/deletions (indels). Wild type *AGXT* comes in two polymorphic variants, the most frequent *major* haplotype (refseq NM_000030) and the less frequent *minor* haplotype, carrying two single amino acid substitutions (P11L and I340M) among other genomic changes in strong linkage disequilibrium. Since these two polymorphisms are quite old, most of the individual PH1 mutations described are typically found in either the *major* or *minor* haplotype, but rarely in both. This fact can help in the clinic, when searching for mutations in new PH1 cases [37]. The *minor* haplotype (simply defined by the refSNP rs34116584, Pro11Leu), with an allelic frequency of 0.1–0.2 in western countries and average heterozygosity around 0.2, does not cause PH1 by itself, but it is known to act synergistically with the deleterious effects of several common mutations [13,38].

2.1.1. Molecular mechanisms of disease

AGXT mutations result in severe reductions of AGT enzymatic activity in the peroxisome, with a relatively wide range of residual activity, depending on the mutations present in both alleles [39]. Although AGT functions as a dimer, all the mutations described so far are related to loss-of-function, with recessive pattern of inheritance, without clear evidence of potential dominant negative effect. Small indels are responsible for some PH1 cases, most notably c.33dupC, the most common mutation of the *major* haplotype, with the predicted consequence of early stop codon and non-sense mediated mRNA decay. But the majority of PH1 alleles are missense mutations, with prevalent amino acid changes that make many PH1 cases good examples of conformational disease. Four main molecular mechanisms have been described to explain the consequences of mutations in *AGXT*: mitochondrial mistargeting, protein aggregation, catalytic defects and synthesis defects.

2.1.1.1. Mitochondrial mistargeting. One of the best studied aspects of AGT is its subcellular localization in various organisms. In mice and rats, AGT is present both in the mitochondria and peroxisomes, while in humans and rabbits, it is exclusively located in the peroxisome [10]. About a third of PH1 alleles involve the G170R substitution, in the *minor* haplotype, which is responsible for mitochondrial mistargeting of the gene product, becoming one of the best known examples of human mutations resulting in mistargeting as the main mechanism of disease [40–42]. In addition to G170R, the missense mutation F152I, also in the minor haplotype was found to cause AGT mistargeting to the mitochondria instead of the peroxisome, leaving the latter organelle devoid of its glyoxylate detoxifying capability. The polymorphism P11L of the *minor* haplotype plays a crucial role for G170R to result in mitochondrial mistargeting [43], which has been associated to impaired folding efficiency to form functional dimers, therefore preventing the peroxisomal import of folded AGT [44]. The mutant protein, particularly in the apoprotein form, devoid of PLP cofactor, has been shown recently to have much reduced kinetic stability *in vitro*, and enhanced interaction with molecular chaperones HSC70 and HSP90 in cell-free assays [45], favoring the presentation of partially folded protein to the mitochondrial import machinery. It has also been shown that the other mistargeting mutation, F152I in the *minor* haplotype, displays substantial kinetic destabilization as apoprotein [46]. Like in other conformational diseases, the partition of mutant AGT between folding into dimers, with subsequent peroxisomal import, and the presentation of partially folded states to the mitochondrial import machinery is likely to depend on various factors in the proteostasis network [47], which are the subject of current investigation.

Protein aggregation is a relatively frequent outcome of missense mutations in conformational diseases [48]. Several of the most frequent PH1 mutations of the *minor* haplotype, such as G41R [41] and I244T [38], are known to display protein aggregation. The P11L polymorphism was also found to play a crucial role for the I244T mutation to result in a conformationally unstable protein, sensitive to proteolytic cleavage and prone to aggregation [38], while the G41R

mutation disturbs the local interactions of the N-terminus, causing conformational instability as well [49].

Catalytic defects are a common mechanism of disease in inborn errors of metabolism involving enzyme-coding genes. Fine enzymatic characterization of several PH1 missense mutations (G82E, G41R, F152I) have demonstrated significant alterations in their performance, including strong decreases in catalytic efficiency and reduced binding affinities for PLP and PMP cofactors [35].

Synthesis defects are mainly represented by small indels with secondary shift in the reading frame and early stop codons. The most common mutation in this group is c.33dupC, the main mutation of the *major* haplotype, present in around 13% PH1 alleles. The stretch of repeated C at the beginning of the coding sequence seems to represent a hotspot for polymerase slippage, where in addition to c.33dupC, c.33delC and c.32_33delCC have also been found [50]. In fact, c.33dupC is the only change for which clear evidence of *de novo* mutation has been reported [51]. Reading frame shifts frequently lead to the appearance of premature stop codons a few bases downstream, and the presence of stop codons in the first few exons normally leads to transcript degradation due to nonsense-mediated mRNA decay [52]. We could also include in this category a large proportion of the reported splicing mutations and occasional missense mutations leading to highly unstable protein that is degraded rapidly, such as S205P [53].

Other mechanisms are involved in a small proportion of PH1 patients, but they also contribute to a better understanding of the disease [54]. Particularly interesting are the cases of Zellweger spectrum disorders (ZSDs), characterized by a deficient peroxisomal assembly and generalized loss of peroxisomal functions. Although most peroxisomal enzymes are unstable in the cytosol of ZSD patients, AGT and a few other enzymes remain stable. However, when carefully examined, most of these patients do develop hyperoxaluria, underscoring the importance of the compartmentalization of AGT in human peroxisomes in order to achieve proper glyoxylate detoxification [55].

Deeper understanding of the molecular mechanisms of disease not only helps derive potential genotype–phenotype correlations but also guides targeted therapeutic approaches (see below). Genotype–phenotype correlations have been described for some mutations of the *AGXT* gene [13,56,57], but the wide allelic heterogeneity limits this type of analysis to the most common mutations. The use of international registries will power this approach to levels that cannot be achieved by single center studies. Significant environmental influences and the potential effect of genetic modifiers also play an important role, to the point that siblings who share the same genotype could have very different clinical phenotypes [58].

2.2. Primary hyperoxaluria type II

PH2, accounting for around 10% of PH cases, is caused by deficient glyoxylate reductase–hydroxypyruvate reductase (GRHPR). A deficit in hydroxypyruvate reductase (HPR) activity as a cause of a new PH variant was first reported in 1968 [27], and it later became evident that the deficiency in glyoxylate reductase (GR) activity, catalyzed by the same protein, was most likely responsible for the hyperoxaluria in this disorder [59]. The lack of GRHPR activities in PH2 patients leads to increases in urinary oxalate and L-glycerate excretion (hence the term L-glyceric aciduria used in the first report) (Fig. 1). PH2 patients suffer less severe elevations of oxalate in the urine than PH1, and their progression to ESRD occurs in a minority of cases. GRHPR is abundant in hepatocytes, and the liver is the main source of oxalate in PH2, but GRHPR is also expressed to some extent in many tissues [60], and its expression in renal tubules is likely to contribute significantly to the mechanisms of kidney damage in PH2.

The *GRHPR* gene is located on chromosome 9 (9q12) and consists of 9 exons, with various mRNA species detected, which encode a 328 amino-acid protein that forms ~73 kDa homodimers. Two groups first identified mutations of this gene as cause of PH2 [61,62], and detected GR, HPR and D-glycerate dehydrogenase (D-GDH) activities in the wild-type gene product. The GRHPR protein can be detected both in cytosolic and mitochondrial fractions, although no clear mitochondrial-targeting sequence (MTS) is present at the N-terminus and alternative routes to the classical presequence-mediated mitochondrial import might be involved.

The structural information derived from crystallized GRHPR has allowed a better understanding of its substrate specificity, NADPH binding, dimerization and other functional aspects of this protein [63].

A current PH mutation database [50] includes less than 30 different PH2 mutations of all types, expected to result in loss of protein expression or catalytic activity. There is a high proportion of small indels, with a single mutation in exon 2, c.103delG, accounting for about 40% of PH2 alleles in populations of north European descent [64,65]. Another common small deletion at the exon/intron 4 boundary, c.403_404+2delAAGT, which results in missplicing, is also relatively frequent (18% of PH2 mutations), while few missense mutations have been reported in more than one family.

The 3D structure of GRHPR has been useful to understand the mechanisms involved in the reported missense mutations with reduced catalytic activity. Among these, G160R and G165D involve residues in the co-enzyme binding groove, M322R most likely destabilizes the channel leading to the active site, and two substitutions at R302 (R302H and R302C) disrupt the interactions with a water molecule and residues S296 and W141 from the adjacent subunit that control substrate access to the active site [63].

2.3. Primary hyperoxaluria type III

PH3 accounts for around 10% of PH cases and it is caused by deficient 4-hydroxy-2-oxoglutarate aldolase (HOGA1), the mitochondrial enzyme that cleaves 4-hydroxy-2-oxoglutarate into pyruvate and glyoxylate, the last step of the hydroxyproline degradation pathway [8]. Since its recent description, the presence of HOGA1 mutations is being investigated in all non-PH1/PH2 patients and PH3 is becoming more frequent than PH2 as a cause of primary hyperoxaluria. PH3 patients show a wide range of oxalate elevations in the urine, and HOGA1 mutations are being found even in patients with milder hyperoxaluria. PH3 progression to ESRD has not been reported. Deficient HOGA1 activity in PH3 patients most likely results in increases in 4-hydroxy-2-oxoglutarate, which could be transformed into glyoxylate by cytosolic aldolases [66], where glyoxylate is later transformed into oxalate by LDH (Fig. 1). HOGA1 is abundant in hepatocytes, and the liver is the main source of oxalate in PH3, but HOGA1 is also present in significant amounts in the kidney, which is likely to contribute significantly to the mechanisms of urolithiasis and renal disease in PH3.

The *HOGA1* gene is located on chromosome 10 (10q24.2) and consists of 7 exons, with a main 2.5 kb mRNA transcript in the liver, which encodes a 327 amino-acid protein that forms ~72 kDa dimers and ~144 kDa tetramers. Although extensive work had been done on the biochemistry of hydroxyproline metabolism for decades [8], and HOGA1 proteins had been purified from mammalian liver and kidney [67,68], it was the detailed genetic analysis of a few PH families in which both *AGXT* and *GRHPR* mutations had been ruled out that recently identified *HOGA1* as the gene responsible for PH3 [69]. Once established that *HOGA1* mutations could account for many of the non-PH1/PH2 cases, the work could proceed quickly to find new mutations in this well-annotated gene among the DNA samples from hyperoxaluric patients without mutations in either *AGXT* or *GRHPR*,

and even among patients with mild hyperoxaluria and idiopathic urolithiasis [66].

The HOGA1 protein is synthesized with an N-terminal MTS of 25 residues, which is cleaved after import in the mitochondria. The structural information derived from crystallized HOGA1 [70] has enabled a deeper understanding of the type I aldolase reaction involved, identifying key interactions for substrate binding, definition of the active site and other functional aspects of this protein. With this structural background, faster and more accurate predictions of the consequences of sequence variants currently detected among non-PH1/PH2 patients will be possible.

In addition to the exon 7 in-frame deletion c.944_946delAGG, common among the Ashkenazi Jewish patients, that played an important role in the identification of the gene responsible for PH3, several missense mutations have been identified recently [66] [Beck et al., Rumsby et al., personal communication]. In line with the autosomal recessive pattern of inheritance, the consensus is emerging that PH3 mutations convey loss of function, with proteins deficient in either dimer/tetramer formation, stability or catalytic activity.

3. Tissue damage in PH1

Elevated oxalate has a major effect on calcium oxalate supersaturation, resulting in the formation of calcium oxalate (CaOx) crystals (both monohydrate -COM- and dyhydrate -COD-). CaOx crystals not only aggregate in the urinary space producing stones (urolithiasis) but they also interact with the renal tubule epithelium and deposit into the renal interstitium (nephrocalcinosis), where they induce strong inflammatory response, often with the formation of "foreign-body" type granulomas (Fig. 2), and progressive interstitial fibrosis that disrupt renal function. The interaction between calcium oxalate crystals and the tubular epithelium seems to be enhanced by the expression of cell surface molecules such as CD44, hyaluronic acid and osteopontin [71]. Although many aspects of the mechanism of tissue damage in PH1 remain unknown, it seems that calcium oxalate crystals [72] rather than the oxalate ion, at the levels found in the tubular fluid [73] are involved in tubular cell damage. In addition to direct tissue damage by calcium oxalate crystals and inflammation, recurrent urolithiasis, with frequent urinary tract obstructions and infections complicate the condition to the point of ESRD. However, patients with early diagnosis, good compliance to conservative treatment and specific genotypes that respond to vitamin B6 administration (see below) may preserve stable renal function.

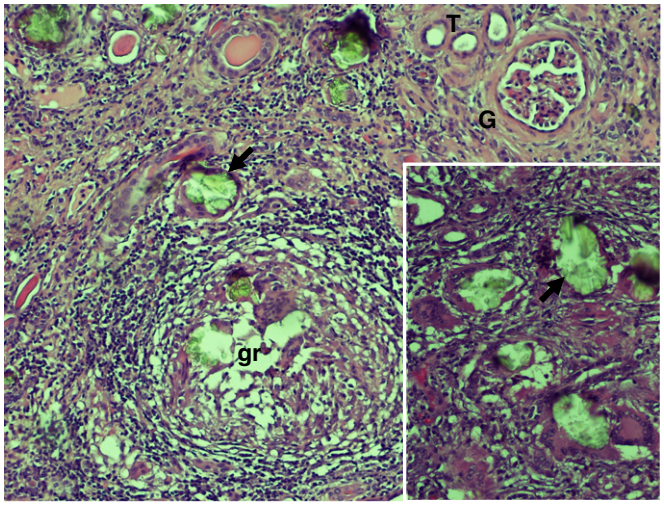

Fig. 2. Nephrocalcinosis. Renal parenchyma damage due to tubular and interstitial deposition of calcium oxalate (arrow). Intense interstitial inflammatory reaction, with formation of foreign-body type granulomas (gr), centered by giant cells engulfing calcium oxalate crystals (inset) and surrounded by epithelioid histiocytes and a collar of mononuclear leukocytes. Widespread interstitial inflammation leads to prominent tubular atrophy (T) and glomerular ischemic changes (G).

Once glomerular filtration rate (GFR) falls below 30–40 mL/min per 1.73 m^2 body surface area, renal excretion of oxalate is insufficient to keep plasma oxalate concentration within the normal limits (below 6 μmol/L [74]) and it can quickly exceed the supersaturation threshold for plasma calcium oxalate, with levels above 30 μmol/L, leading to widespread deposition of calcium oxalate (oxalosis). At this stage, in addition to the kidney, many organs are damaged by calcium oxalate crystals and the inflammatory response around them. Retina, myocardium, vessel walls, skin, bone, and the central nervous system are the main organs affected by oxalosis, and long-term complications of PH include cardiomyopathy, cardiac conduction disturbances and heart block, vasculopathy, treatment-resistant anemia, oxalate osteodystrophy, retinopathy and early death [17,18].

4. Clinical aspects of PH

Although the metabolic defects in PH is present from birth, there is a striking variability in the age of presentation of symptoms, ranging from severe infantile oxalosis (reported only in PH1) to adult presentations that resemble idiopathic kidney stone disease [17,22].

The age of presentation is typically the first decade, and the most common first symptoms are hematuria, abdominal pain, passage of a stone or repeated urinary tract infections. In the more severe infantile forms, patients present with signs and symptoms of renal failure already, with failure to thrive, anemia and acidosis.

In adults, PH1 often goes unrecognized for years, with patients considered to suffer idiopathic kidney stone disease, until severe kidney damage (nephrocalcinosis) has already occurred [75].

Progressive renal interstitial inflammation and fibrosis related to the parenchymal calcium oxalate deposition, recurrent urolithiasis and urinary tract infections, ultimately lead to ESRD and subsequent systemic oxalosis. At this point, the diagnosis of PH may be overlooked and ESRD attributed to idiopathic stone kidney disease. As for PH2, a minority of patients (around 20%) develop ESRD, while no progression to this stage has been reported for PH3.

In general, PH1 has a poor prognosis unless liver (and kidney) transplantation are performed, mostly due to cardiovascular complications of systemic oxalosis after renal failure. In a recent analysis of the European registries (ESPN/ERA-EDTA), among 9247 incident pediatric patients that started renal replacement therapy (RRT) in the 1979–2009 period, 100 had PH, and their survival 5 years after the start of RRT was only 76% [14]. This figure represents a three-fold higher mortality than non-PH patients on RRT. For infantile PH1, which is often diagnosed when the patient has lost renal function, a survey of specialized centers worldwide showed 52% mortality: 82% in developing countries *vs.* 33% in developed ones [76].

Early diagnosis and treatment are crucial for PH patients, as shown by recent clinical studies that found median ages at ESRD above 30 years old now, while earlier series reported a 50% risk of ESRD by the age 15, and about 80% by age 30 [21,22,77]. Although the mortality rate can be reduced, patients are still at high risk of developing side effects of the symptomatic treatment. PH1 patients generally have a poor quality of life unless liver–kidney transplantation is successful. Even in this situation, the patient is dependent on immunosuppression and the life expectancy is significantly reduced. Only long term correction of the defect and prevention of kidney damage can reasonably be expected to improve such consequences.

In contrast to PH1, which can be as severe as to cause ESRD in infants, PH2 appears to have less severe tissue damage and better preservation of renal function over time [78]. Although the clinical experience with patients diagnosed with PH3 mutations is still limited, it seems to be also milder than PH1, and high calcium and uric acid, in addition to high oxalate, tend to be present in their urine [66,69].

5. Diagnosis

Early diagnosis and therapy are crucial to preserve renal function in PH patients and delay ESRD as much as possible. PH should be investigated in patients with nephrocalcinosis and kidney stone disease, after ruling out common causes of these entities. PH should also be considered in all cases of familiar stone disease and renal failure of unknown cause.

Elevated urine oxalate (UOx) excretion > 0.5 mmol/1.73 m^2 per day (typically > 45 mg for an adult) is characteristic of PH, provided that repeated measurements are consistent, and secondary hyperoxaluria has been ruled out. In PH1 patients, overproduction of oxalate by the liver results in very high urinary oxalate excretion, typically > 1 mmol/1.73 m^2 per day, while less severe hyperoxaluria is normally found in PH2 and PH3 patients. Confirmation of hyperoxaluria from a 24 hour urine collection related to body surface area is recommended. In children, where 24 hour urine collection is not practical, oxalate/creatinine ratio can be determined on random urine specimens. Ratios fall rapidly in early life and are influenced by prematurity and nutrition; thus interpretation requires an age-related reference range [79]. A raised urine glycolate excretion is suggestive of PH1 but it is elevated in only two thirds of patients and its levels vary depending on diet. Elevated L-glycerate is indicative of PH2.

Plasma oxalate (Pox) will increase as GFR falls, and the rate of increase is much faster than in ESRD due to any other cause, with values above 50, even 100 μmol/L.

The presence of abundant oxalate crystals in a renal biopsy is typical of PH (Fig. 2). Although calcifications in tubular profiles can be found in renal biopsies unrelated to PH, the presence of abundant deposits, particularly if found in the interstitium, with strong inflammatory reaction, should prompt further investigations. Foreign-body type granulomas around calcium oxalate deposits are strong indicators of PH. In some instances, the renal biopsy provides the first clue for PH in patients that had been treated as common kidney stone disease sufferers or, more dramatically, after a renal transplant with delayed function [19]. In these cases, oxalate deposits appear rapidly in the transplanted kidneys and the organ could be lost in weeks or a few months. In advanced cases of uremic bone disease, with radiological evidences of osteitis fibrosa and osteosclerosis, the bone biopsy may provide the definitive diagnosis, showing the typical granulomatous reaction surrounding oxalate deposits.

The measurement of catalytic activities in a liver biopsy is considered the diagnostic gold standard, with a sensitivity > 95% [80], although false negatives are possible, given the wide range of residual activity seen [39]. This is particularly relevant in the case of mistargeting mutations, where AGT with significant enzymatic activity is present in the mitochondria. Part of the liver biopsy can be used for immunohistochemical analysis, to ascertain subcellular localization, and a small amount of tissue is enough for mRNA isolation, but these techniques are not done routinely in most diagnostic laboratories.

On the other hand, less invasive DNA analysis is becoming the first choice for diagnosis in most cases, and it is also used for prenatal testing and diagnosis in other family members after the mutation is known in the index case. In most hospitals, liver biopsy is currently used only in patients for which no mutation is found in the *AGXT*, *GRHPR* or *HOGA1* genes, in order to completely exclude the known variants of PH. There are isolated instances in which hyperoxaluria is due to endogenous overproduction (PH), and the three known genes have been ruled out, but the causal gene has not been found yet. Genome-wide linkage studies are ongoing to try to map such gene(s).

An algorithm to assist in diagnosis has been published [81] and current European guidelines have been drafted by a panel of experts.

6. Prevention

All forms of PH described to date are hereditary, with an autosomal recessive pattern of inheritance. Heterozygous carriers of the mutated genes have, for the most part, no clinical signs or symptoms of the disease. However, carriers of *HOGA1* mutations have been recently identified among patients with mild hyperoxaluria and urolithiasis [66]. Genetic or presymptomatic counseling is only feasible after the diagnosis of an index patient has been confirmed in a family member.

7. Treatment

Conservative measures should be initiated as soon as possible with the goal of preserving renal function. The following measures apply to all types of PH with the exception of pyridoxine which is specific to PH1.

High fluid intake has been proven to be effective in kidney stone diseases [82]. In PH, the recommended fluid intake is at least 3 L/m^2 per day, distributed throughout 24 h. A feeding or gastrostomy tube is often required in infants and small children to achieve this goal. Special care should be taken in situations of fluid losses (diarrhea, vomiting, and fever) or limited oral hydration (surgery) and i.v. fluid intake might be necessary to keep high urine flow. It is important to realize that maintaining a high urine flow is an efficient way to eliminate oxalate, while a temporary flow reduction can lead to calcium oxalate deposits that favor further aggregation of crystals.

7.1. Vitamin B6

PLP, a form of vitamin B6, is a cofactor for AGT. Administration of pyridoxine hydrochloride has been known for a long time to be associated with a decrease in urinary oxalate (UOx) in about 30% of patients with PH1 [83,84], but the cellular and molecular basis of pyridoxine responsiveness is not completely understood. Indeed, this is one of the most intriguing aspects of PH1, that has prompted great hopes for a potential therapeutic effect based on chemical and pharmacological chaperones (see below).

All PH1 patients should be tested for pyridoxine responsiveness, and if responsive, treated until liver transplantation is performed, even if undergoing haemodialysis (HD). The recommended starting dose is 5 mg/kg per day, increasing in 5 mg/kg steps to a maximum of 20 mg/kg body weight per day [85]. Responsiveness is currently defined by > 30% decrease in UOx excretion after a test period of a minimum of 3 months at maximum dose [12,17]. Since vitamin B6 is very safe, with a small risk of sensory neurotoxicity as the main side effect, a trial of pyridoxine treatment should be performed in most cases, and particularly in patients with missense mutations. A subset of patients carrying one or two copies of G170R or F152I mutations have been shown to respond best to pyridoxine [86,87].

Binding of PLP to AGT protein enhances its kinetic stability, increasing its half-life for irreversible denaturation, as shown by differential scanning calorimetry (Fig. 3) and the improvement is highest with some mutant forms of AGT, particularly those in the minor haplotype, such as G170R and I244T, among others, although many mutations tested and even wild type AGT also benefit in their kinetic stability upon PLP binding [45]. It is likely that the most relevant parameter to estimate the kinetic stability of cytosolic AGT folded dimers is the half-life for irreversible denaturation of the apo-forms. Moreover, since PLP binding to AGT is a slow bi-molecular process [3,45], and the total PLP concentration in liver is of the same range of the total AGT concentration, an increase in cytosolic levels of free PLP will significantly speed up the formation of holo-proteins [45], trapping the AGT in a folded and kinetically stable holo-form ready for peroxisomal import. This is specially relevant for PH1 missense mutations which are more destabilized in their apo-form than in their holo-form. In this situation, PLP would preferentially stabilize

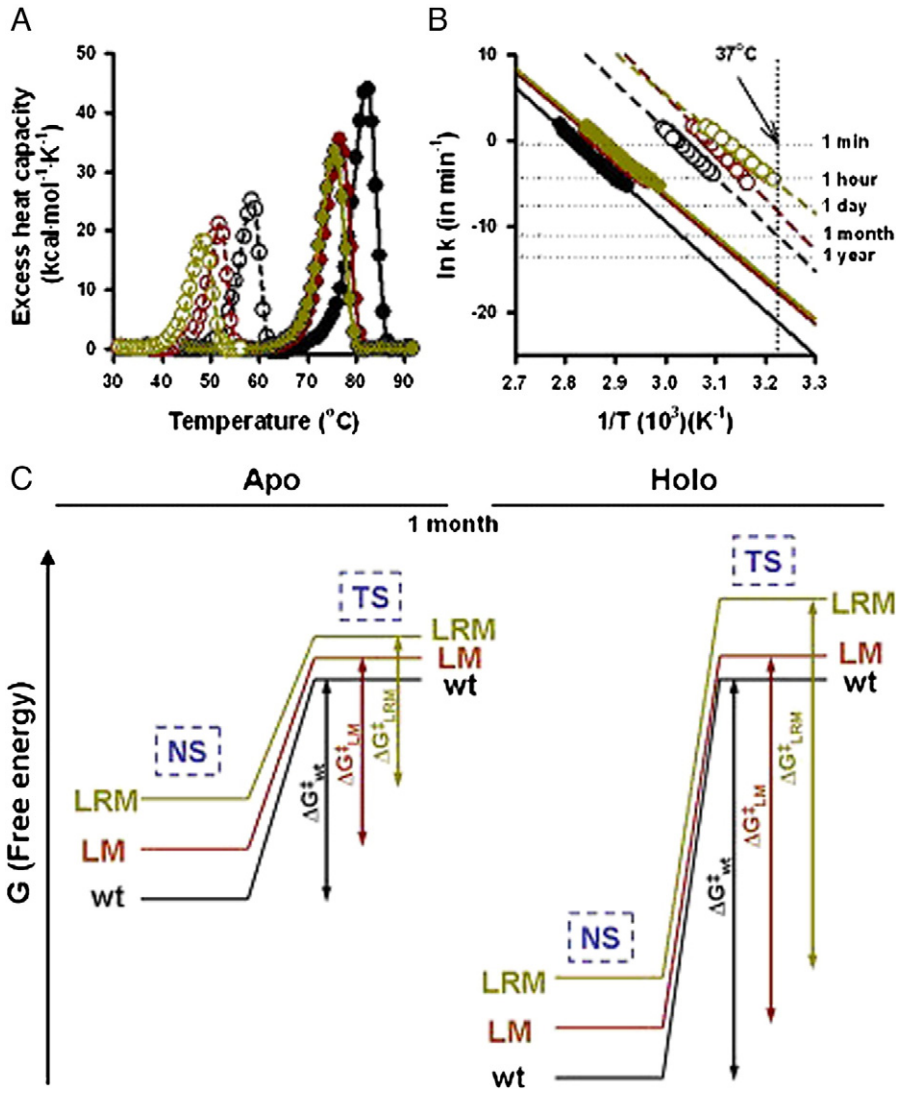

Fig. 3. Effect of PLP binding on the kinetic stability of dimeric AGT-His_6 variants. A) Differential scanning calorimetry scans for holo (closed symbols) and apo (open symbols) variants of wt (black), LM (dark red) and LRM (dark yellow). Lines are fits to a two-state irreversible denaturation model. B) Plots of the natural logarithm of the first-order rate constants (k) for the irreversible denaturation of the AGT variants shown in panel A (Arrhenius plots). Note that the values of k extrapolated at physiological temperature are similar for holo-LM and holo-LRM, while the k values for apo-LM indicate a higher kinetic stability (~25-fold) than the apo-LRM. The values of k corresponding to different half-lives (from 1 min to 1 year) are also indicated. C) Pictorial representation of the possible energetic impact of PLP binding on the free energy levels of the native state (NS) and the denaturation transition state (TS), whose free energy differences ($\Delta G^{\ddagger}$) determine the kinetic stability of AGT variants. Note that the $\Delta G^{\ddagger}$ for apo-proteins decrease as follows: apo-wt > apo-LM > apo-LRM variant, reflecting decreasing kinetic stabilities. However, PLP binding increases the $\Delta G^{\ddagger}$ of holo-LRM more than for holo-LM, leading to similar kinetic stabilities for these two variants as holo-proteins. wt = wild type *major* AGT; LM = *minor* AGT (with polymorphisms P11L and I340M); LRM = mutant G170R AGT in the *minor* haplotype; PLP = pyridoxal phosphate; apo- = AGT without PLP cofactor; holo- = AGT with PLP cofactor.

the native state and/or destabilize the unfolding transition state for irreversible denaturation (Fig. 3C).

Alkalinization of the urine with alkali citrate can reduce urinary CaOx saturation. Liver conversion of citrate to bicarbonate leads to higher urinary pH and subsequent increase in citrate excretion. Citrate then forms complexes with calcium, decreasing CaOx precipitation [88]. Potassium citrate (or sodium citrate, depending on renal function and serum potassium) at a daily dose of 0.10–0.15 g/kg body weight is recommended.

7.2. Diet

The main source of oxalate in PH is the endogenous production by the liver, making strict dietary restrictions on oxalate-containing foods unnecessary. In fact, intestinal oxalate absorption is lower in PH patients compared to normal subjects [15]. Calcium intake should remain normal as oral calcium binds intestinal oxalate and dietary calcium restriction results in higher oxalate intestinal absorption [89,90]. Excessive intake of vitamins C and D is to be avoided, and vitamin D supplementation in children should be used carefully. Given the high hydroxyproline content of gelatin and meats, it seems reasonable to advice their consumption reduction among PH patients, particularly PH3, but no formal recommendation has been included in clinical guidelines.

7.3. Dialysis

Both peritoneal dialysis (PD) and hemodialysis (HD) have been used either alone or in combination in order to maximize oxalate removal [91,92]. Oxalate is a small molecule (90 Da) that can be filtered readily, but oxalate is produced by the PH liver at a daily rate higher (4–7 mmol/1.73 m^2 per day) than the oxalate removing capacity of dialysis (1–4 mmol/1.73 m^2), resulting in oxalate accumulation [91,93]. Thus, dialysis is used while waiting for organ transplantation or achieving adequate body size, but it is not adequate replacement therapy for patients with systemic oxalosis who have reached ESRD

[18,94]. The threshold of CaOx supersaturation ($\beta_{CaOx} > 1$) ranges between 30 and 45 μmol/L, also depending on serum calcium concentration [74,95]. Intensive dialysis is needed to lower plasma oxalate (POx) enough to keep $\beta_{CaOx} < 1$ as long as possible during the interdialytic period. Although a HD session can reduce POx by about 60%, oxalate turnover from tissue deposits and endogenous oxalate production bring POx back to 80% of the pre-dialysis levels within 24 h [96].

7.4. Transplantation

Once a firm diagnosis of PH1 is established, organ transplantation, replacing the oxalate-producing liver with a donor organ, should be planned and best results are achieved if this occurs before systemic oxalosis. The strategy of liver–kidney transplantation is influenced by the stage of the disease [97]. Pre-emptive isolated liver transplantation (LTx) may be an option in selected patients [98,99] but in most cases, the liver is replaced only after sufficient kidney damage has occurred, opting for combined liver–kidney transplantation (LKTx). LKTx has been used successfully with excellent outcome even in small infants [100,101]. A sequential procedure (first liver transplantation, then dialysis until sufficient oxalate has been cleared from the body, followed by kidney transplantation) may be proposed in individual ESRD patients.

Isolated kidney transplantation might be a solution in some patients with good response to conservative measures and vitamin B6 administration, but it is not recommended in the majority of PH1 patients, given the high risk of quick deterioration of the renal allograft, still overloaded with oxalate produced by the PH1 liver [19]. In milder PH variants (most cases of PH2, and probably PH3), kidney transplantation might be sufficient to control the disease, but there are not evidence-based guidelines available yet.

8. Prospects for molecular therapy

Significant advances in the pathophysiology of PH in the last couple of decades have led to better diagnosis and treatment of these patients, but current therapeutic options are far from ideal. The risks associated with liver transplantation (survival rates of 86 and 69% at 1 and 10 years, respectively [102]) make it difficult, for patients, families and doctors, to go ahead and replace an otherwise healthy liver from a PH1 patient in order to save the kidneys. Thus, LTx is usually delayed until complicating renal failure necessitates LKTx [103]. In addition, there are significant health risks associated to long-term immunosuppression, with 13% and 31% of patients reaching stage 3 chronic kidney disease at 5 and 10 years after LTx, respectively [104]. The situation in PH1 is even worse [105], most likely due to the synergistic nephrotoxic effect of immunosuppression and oxalate.

Although PH is a rare disease, there is considerable interest in translating the recent advances in the understanding of the molecular mechanisms of disease into better targeted therapies [106]. Some of these efforts can be implemented *in vitro*, but the development of genetic animal models for PH [107] (and unpublished) has provided excellent testing grounds for *in vivo* experimentation.

8.1. Oxalate degrading enzymes

Oxalate cannot be metabolized in mammals, but other forms of life have enzymes such as oxalate oxidase and oxalate decarboxylase, capable of degrading oxalate. These enzymes have been investigated as a novel solution to oxalate accumulation in PH. It has also become clear that some oxalate can be eliminated by the gastrointestinal tract, using the anion exchanger SLC26A6 [108], and this route could be relevant when renal function deteriorates. A mouse *Slc26A6* deficient model has been shown to develop severe hyperoxaluria [109], bringing more attention to this potential therapeutic target, although significant differences between human SLC26A6 and its rodent counterpart have to be considered [110]. Recombinant, stabilized, oxalate oxidase has been tested in hyperoxaluric mice, showing a significant reduction in urine oxalate excretion [111]. Strategies to increase oxalate degradation by gut colonization with the oxalate-metabolizing bacteria *Oxalobacter formigenes* have shown promising results in the mouse PH1 model [112] and in some pilot studies with PH1 patients [113], but a consistent benefit derived from the use of probiotics with *O. formigenes* to reduce oxalate load in PH1 patients could not been demonstrated so far [114], and new delivery options continue being investigated.

8.2. Enzyme replacement therapy (ERT)

Remarkable improvements have been achieved by ERT in some inborn errors of metabolism, particularly the lysosomal storage diseases [115]. This approach has not been tested in PH, deterred by the very significant challenges unique to PH1. The fact that any hepatocyte that is not corrected will continue producing oxalate poses an important limitation to strategies aimed at correcting hepatocytes by delivery of enzymes or genes. In addition, proteins are taken up by hepatocytes via endosomes, and the replaced AGT would have to be efficiently targeted to the peroxisome. Advances in the field of cell-penetrating peptides and subcellular targeting could bring the ERT option to the PH testing arena in the future.

8.3. Gene therapy (GT)

After years of ups and downs in the field, recent developments have provided renewed optimism for approaches to treat hereditary enzyme deficiencies by providing the normal sequence of the gene to the key defective cells [116]. Again, in a disease like PH, where non-transduced hepatocytes would continue to produce oxalate, the therapeutic goal can only be achieved if sufficient hepatocytes are transduced so that the overall production of oxalate by the treated liver remains below the capacity of the kidneys to excrete it without suffering irreversible damage. The development of viral vectors with enhanced hepatocyte tropism and minimal immune response results in high proportions of hepatocytes showing sustained expression levels, making gene therapy a realistic option for PH in the future. In fact, using adenoassociated viral particles, the treatment of the PH1 mouse model has been quite successful [117].

8.4. Substrate reduction therapy (SRT)

The main sources of glyoxylate are glycine, glycolate and hydroxyproline, in metabolic reactions catalyzed by enzymes such as D-amino oxidase (DAO), glycolate oxidase (HAO1) and 4-hydroxy-2-oxoglutarate aldolase (HOGA1). Thus, a therapeutic strategy to ameliorate glyoxylate build-up in PH patients would be to inhibit any non-essential pathway leading to glyoxylate. The idea of inhibiting HAO1 as substrate reduction therapy is anything but new. The structure of the spinach homolog of human HAO1 has been available for years, and small molecule inhibitor of this enzyme had been found, but the results of a pilot study in PH1 patients were disappointing [118]. The biochemical and structural properties of human HAO1 has been described recently [119,120]. It seems plausible that renewed attempts to inhibit HAO1 with increasing specificity will eventually result in a useful SRT for PH1. In addition to increased specificity of the small molecules, avoiding inhibition of other FMN-dependent enzymes, for this SRT strategy to be safe HAO1 must play non-essential roles in human cell metabolism. Using genetically modified mice, we have observed that *Hao1* deficient mice are healthy and the introduction of this second KO gene in the PH1 mouse model results in a dramatic reduction in urinary oxalate excretion (manuscript in preparation). Metabolic differences between

human and mice might have an impact on the expectations raised by these results, but the data currently available points to HAO1 as a good target for SRT in PH1.

In theory, DAO could also be a potential target for SRT, and there is interest in the pharmaceutical industry to develop DAO inhibitors, given the implications of this enzyme in mental diseases [121].

With recent evidence that hydroxyproline is an important source of glyoxylate in humans [5], and the discovery of HOGA1 as the gene responsible for PH3 [69], this pathway is increasingly attractive as a target for SRT. Two enzymes are specific to hydroxyproline degradation: hydroxyproline dehydrogenase (oxidase) (PRODH2) and HOGA1. Although HOGA1 seemed a plausible target at first, recent evaluation of mutations in PH3 patients indicates that inhibiting HOGA1 would increase, rather than decrease, oxalate production. On the other hand, PRODH2, the first enzyme in the mitochondrial hydroxyproline degradation pathway remains a good candidate for SRT in PH in general, and PH3 in particular. By using genetically modified mice, we are currently testing whether PRODH2 is a safe and efficient target.

8.5. Chemical chaperone and proteostasis regulation therapy (CC–PRT)

"Proteostasis" is an integrative definition of protein intracellular homeostasis which combines concepts from protein folding and stability, cellular macromolecular assemblies assisting protein folding, degradation and intracellular trafficking, and systems regulating protein expression and function at transcriptional, translational and post-translational levels. This complex and highly regulated network determines the intracellular fate of proteins [47,122]. Recent studies have shown that AGT native state kinetic stability [45,46] and interactions of partially folded states with molecular chaperones such as Hsp40/DnaJ (unpublished), Hsp60/GroEL, Hsc70 and Hsp90 [38,45,123] may be involved in the partition of AGT protein between correctly folded dimers and misfolding, with mitochondrial mistargeting, aggregation and degradation.

Therefore, several proteostasis elements emerge as potential targets for therapeutic intervention in PH1. Moreover, a recent report suggest that the cytosolic levels of folded AGT may be a relevant factor determining the efficiency of its peroxisomal import, due to the very low affinity displayed for the PEX5 receptor compared to other peroxisomal proteins [33].

Pharmacological modulation of molecular chaperones is a promising therapeutic strategy in several conformational diseases [124]. The canonical model for the HSP70 machinery has been elucidated with great detail in prokaryotes, but the system is much more complex in humans, displaying at least 11 HSP70s, 41J-proteins and 13 nucleotide exchange factors (NEFs) [125]. In addition, mammalian J-proteins and NEF proteins are structurally and functionally complex, displaying additional functions beyond the canonical model. For instance, J-proteins are known to be involved in shunting client proteins for degradation, remodeling and partially unfolding client proteins, while the BAG (BCL2-associated athanogene) family of NEFs are also known to target client proteins for proteasomal degradation [125]. Therefore, finding targets in the HSP70 machinery for therapeutic intervention in PH1 requires an exhaustive biochemical and cellular research on all these targets to determine those responsible for mitochondrial mistargeting, aggregation and degradation.

A different approach to treat PH1 could be based on the kinetic stabilization of the AGT native state with pharmacological or chemical chaperones. Pharmacological chaperones are small-molecules which specifically bind to the protein native state leading to either thermodynamic or kinetic stabilizations. PLP (see above) is a special case of natural kinetic stabilizer of AGT (Fig. 3) [35,45]. However, the basic mechanism of PLP kinetic "overstabilization" of missense mutations is not fully understood. According to the transition state theory, denaturation rates are determined by the activation free energy, the free energy difference between the native and the unfolding transition state (which is the unfolding rate limiting step and not directly accessible to investigation). Thus, PLP binding must increase the unfolding free energy barrier in some mutations compared to the wild type enzyme. Our previous analyses have shown that PLP induced notable kinetic stabilization of wild type AGT, increasing the unfolding free energy barrier by 6–7 kcal/mol at 37 °C, but still lower than the free energy of PLP bound to the native state (around 10 kcal/mol at physiological temperature [45]). This suggests that PLP remains bound to the unfolding transition state but possibly with *lower affinity*. Thus, it is plausible that PLP binding partially overcome mutation-induced destabilization of the native state, while these PLP effects are marginal or not observed at all in the unfolding transition state. The unfolding transition states for these mutants might show some distortion in PLP binding compared to the wild type protein. Nevertheless, a throughout investigation of mutational effects on the structure and energetics of the native and transition states must be carried out to dissect these differences found between holo- and apo-AGT in some variants, as well as to evaluate the impact of mutations at different AGT domains and structural positions.

Protein stabilization is easily adapted to high-throughput screening of chemical libraries based on a thermal up-shift assay to find potential pharmacological chaperones [126]. In some cases, pharmacological chaperones resemble known protein ligands or inhibitors, and their stabilizing effect may be enhanced by structure-based approaches [127]. The efficacy of pharmacological chaperones may be sensitive to mutations, since they may affect binding affinities, folding/unfolding mechanisms and interactions with the proteostasis network. Consequently, pharmacological chaperones have been shown to correct protein misfolding in several genetic diseases and it seems likely that missense PH1 mutations can benefit from this approach in the future. Chemical chaperones are small organic compounds which favor compact protein states over unfolded states through the so-called "solvophobic effect", which involves destabilizing interactions of the water/chaperone mixture with the polypeptide backbone [128]. Their beneficial effect on mutant AGT has been demonstrated *in vitro*, [38,49] but the main drawback of these compounds are the high concentrations required for protein stabilization and the unspecificity of the stabilizing effect, potentially affecting the physiological turnover of multiple proteins.

8.6. Cell therapy (CT)

Liver transplantation has changed dramatically the outcome of many inborn errors of liver metabolism [129], and PH1 is a good example. However, the morbidity and mortality associated with this procedure is significant, and alternatives to total liver transplantation have been explored in order not to make the recipient's life absolutely dependent on the metabolic competence of the allograft. But partial liver transplantations have failed to provide enough reduction of oxalate production in PH1 [102]. Hepatocyte cell transplantation is a promising alternative that is being actively explored in inborn errors of liver metabolism [130], including PH1, at the experimental level [131,132].

Hepatocyte transplantation is a minimally invasive procedure, although the number of mature hepatocytes that can be safely injected in one session is not enough to treat standard autosomal recessive enzymatic deficits [133], and probably less so for PH1 in particular.

In cases when the healthy hepatocytes from the donor have a selective advantage over the recipient ones, the regenerative potential of hepatocytes can be used to achieve gradual repopulation of the liver, an approach that has been successful in mouse model of hereditary tyrosinaemia type I [134]. Interestingly, this model has been improved to be able to use induced pluripotent stem cells (iPS) derived from fibroblasts, to generate the hepatocyte-like cells that could be transplanted to repopulate the diseased liver [135]. A major obstacle

to the translation of these approaches to PH is that native hepatocytes produce large amounts of oxalate but do not seem affected by it, and they are not at a selective disadvantage with respect to the transplanted cells. From our gene therapy experiments with the PH1 mouse model, we calculated that around 40% hepatocytes need to be transduced with the wild type gene to revert the hyperoxaluric phenotype [117]. Replacing a similar proportion of hepatocytes, which in the human liver would represent about 2×10^{10} cells, cannot be achieved without manipulations that provide selective advantage to the transplanted cells. In previous experimental studies, we have shown that liver irradiation can be used to limit the regenerative capacity of the endogenous hepatocytes, selecting for the transplanted cells to be the only ones responding to proliferative stimulus, and repopulating the liver over time [131,132]. Alternative methods, based on pro-drugs that become hepatotoxic only after reacting with high levels of cytoplasmic glyoxylate would be useful.

Combining the concepts of gene and cell therapy, one can envision a situation in which iPS cells can be derived from PH1 fibroblasts, their *AGXT* gene corrected *in vitro*, and later differentiated into hepatocytes for autologous cell transplantation. The gene correction part of the procedure could be implemented with novel nuclease-based targeted strategies [136], avoiding the need to rely on homologous recombination for gene correction, and opening the possibilities to efficient gene correction in non-ES cell types, such as fibroblasts, as recently shown in mouse experimental models [137].

Aknowledgments

This work was supported by grants from the Spanish Ministry of Science and Innovation (SAF2007-62343, SAF2011-23933, FIS07/963, BIO2009-09562, CSD2009-00088 and RyC2009-04147).

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