Biomedical Genetics and Genomics

Review Article

ISSN: 2398-5399

Protective mechanisms against protein damage in hyperhomocysteinemia: Systemic and renal detoxification of homocysteine-thiolactone

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Abstract

Homocysteine (Hcy) and its metabolite Hcy-thiolactone (HTL) are implicated in cardiovascular disease (CVD). Recent studies show that HTL is a predictor of acute myocardial infarction in CVD patients, independent of established risk factors and plasma total Hcy. HTL is formed in all cell types as a result of error-editing reactions in protein biosynthesis. Its ability to N-homocysteinylate protein lysine residues and cause protein damage has been mechanistically linked to the pathology of CVD induced by hyperhomocysteinemia. Specific HTL-detoxifying mechanisms have been identified that can potentially be exploited for modulation of HTL levels and the risk of CVD.

Introduction

Traditional risk factors account only for half of cardiovascular disease (CVD) cases. Thus, there is a need to study other CVD risk factors and elucidate their mechanism of action. Mildly elevated plasma total homocysteine (tHcy) is an emerging risk factor [1] that is associated with increased risk of CVD and is a strong predictor of mortality in cardiovascular patients [2]. Folic acid and B-vitamin supplementation lowers plasma tHcy and has been studied for primary and secondary prevention of CVD outcomes in large-scale randomized controlled trials (RCTs). In individual RCTs, lowering plasma tHcy by folic acid and B-vitamin supplementation protects against stroke [3,4], but not myocardial infarction [5,6]. However, meta-analyses of 8 RCTs involving 37,485 individuals [7] completed by the end of 2009 and 24 RCTs involving 57,952 individuals [8] completed by April 2013 show that tHcy-lowering by B-vitamin supplementation has no effect on CVD outcomes.

A possible reason for these dissonant results is that tHcy is a composite marker comprising of different Hcy species, but not encompassing a chemically reactive and toxic Hcy metabolite—Hcy-thiolactone (HTL)—which has been independently implicated in CVD [9]. Indeed, recent studies show that HTL is a predictor of acute myocardial infarction in CVD patients, independent of established risk factors and plasma total Hcy [10]. Moreover, HTL levels are not reduced by folic acid/vitamin B_{12} treatments that lower Hcy [10]. HTL, produced in an Hcy-editing reaction during protein biosynthesis [11,12], can promote CVD due to its ability to form isopeptide bonds with protein lysine residues, which generates toxic *N*-Hcy-proteins with pro-inflammatory, pro-thrombotic, pro-atherogenic, and pro-amyloidal properties [9].

Because HTL accumulation compromises biological integrity, humans and animals have evolved mechanisms to eliminate or detoxify HTL. One such mechanism involves enzymatic hydrolysis by HTL hydrolases (HTLases): serum HTLase/paraoxonase 1 (PON1) [13], cytoplasmic HTLase/bleomycin hydrolase (BLMH) [14], and mitochondrial HTLase/bisphenol hydrolase-like (BPHL) [15-17].

Paraoxonase 1

PON1 is a calcium-dependent enzyme synthesized in the liver and circulating in the blood attached to high-density lipoprotein HDL; it is the first well-characterized HTLase [13,18]. Pon1, named for its ability to detoxify the organophosphate paraoxon, is implicated in CVD and Alzheimer's disease (AD). For example, low serum Pon1 activity is a risk factor for dementia [19] and AD [20,21], while Hcy is a negative determinant of Pon1 activity [22,23] and a risk factor for AD [24]. Emerging evidence strongly suggests that PON1 activity is linked to CVD risk. For instance, PON1 protects against (high-fat diet-induced) atherosclerosis in humans [25,26] and mice [27]. The cardio-protective function of PON1 has been mechanistically linked with its ability to modulate indices of oxidative stress [26,27] and to detoxify HTL [13,28,29]. HDL and purified PON1 have the ability to hydrolyze HTL [13] and to protect against the accumulation of *N*-Hcy-protein *in vitro* [30,31] and *in vivo* in humans [28].

Substrate specificity studies of purified human serum PON1 show that *L*-HTL is a preferred physiological substrate [13] (Table

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Keywords: *atherosclerosis, homocysteine thiolactone, PON1, BLMH, BPHL, renal function*

Received: June 01, 2016; **Accepted:** June 25, 2016; **Published:** June 28, 2016

1). Non-physiological substrates *D*-HTL and γ-thiobutyrolactone are hydrolyzed at a rate 4-fold slower and 5.5-fold faster, respectively, than *L*-HTL by the enzyme. Other non-physiological substrates phenyl acetate and paraoxon (hydrolyzed 2,800- and 3.3-times faster that r*L*-HTL) are non-competitive inhibitors of the HTLase activity suggesting that HTL, phenyl acetate, and paraoxon are hydrolyzed at different sites of the enzyme [13]. This suggestion is supported by structure/function studies showing that specific active sites mutations have different effects on arylesterase, paraoxonase, and lactonase activities of the PON1 protein [32,33]. Other inhibitors of the HTL activity, such as isoleucine and penicillamine are also non-competitive, suggesting the presence of a distinct amino acid-binding effector site on PON1 [13]. HTLase and paraoxonase activities are strongly correlated in various populations [22,28,31], indicating that the non-physiological paraoxonase activity is a good surrogate for the physiological HTLase activity of the PON1 protein.

Human *PON1* has two major genetic polymorphisms: *PON1-M55L* and *PON1-R192Q*, which affect PON1 function [34,35], including its HTLase activity [22,31]. For example, high HTLase activity is associated with *PON1-L55* and *PON1-R192* alleles, whereas low HTLase activity is associated with *PON1-M55* and *PON1-Q192* alleles [22,31]. Purified serum PON1-R192 allozyme has 2.5-fold higher HTLase activity than the PON1-Q192 allozyme [36], which explains the association of high activity with *PON1-R192* allele. However, several studies have found that PON1 phenotype (HTLase or paraoxonase activity) is a predictor of cardiovascular disease but the *PON1-R192Q* or *PON1-M55L* genotypes are not [22,34,35,37-39]. For example, HTLase activity is found to be significantly lower in a group of 128 CVD patients with angiographically confirmed atherosclerotic lesions, compared to a control group of 142 individuals who have no lesions [39]. A negative correlation is found between the severity of atherosclerotic lesions and HTLase activity in patients. Further, the physiological HTLase activity is a more significant predictor of CVD than a non-physiological paraoxonase activity [39].

In humans, the HTLase activity (but not arylesterase or γ-butyrothiolactonase) of PON1 is a determinant of plasma *N*-Hcy-protein levels and protects proteins against *N*-homocysteinylation *in vivo*, a novel mechanism likely to contribute to atheroprotective roles of HDL [18,28]. HTLase activity of PON1 is negatively correlated with tHcy [22] and predicts CVD (38). *Pon1*$^{-/-}$ mice are impaired in their ability to hydrolyze HTL and as a result heve elevated levels of plasma *N*-Hcy-protein and excrete more HTL in the urine compared with their *Pon1*$^{+/+}$ littermates [29]. *Pon1*$^{-/-}$ mice are also more sensitive than their wild type littermates to neurotoxicity of HTL. Taken together, these studies provide the first direct evidence that a specific Hcy metabolite, HTL, rather than Hcy itself is neurotoxic *in vivo* [29], and suggest that other functional properties of HDL beyond its ability to promote reverse cholesterol transport contribute to its atheroprotective function.

Bleomycin hydrolase

Bleomycin hydrolase (Blmh), is a thiol-dependent cytoplasmic aminopeptidase expressed in various organs, including the liver [40]. Blmh is studied in the context of Hcy toxicity [14,41], cancer therapy [42,43], AD [44-47], Huntington disease [48], keratinization disorders [49], and protein breakdown [48,50]. The human genetic polymorphism *BLMH-Ile443Val* is associated with an increased risk for AD [45]. In mice, deletion of the *Blmh* gene results in several phenotypes, such as neonatal mortality, tail dermatitis [51], brain pathology [52], and impairs the presentation of some antigens [53].

In addition to an aminopeptidase activity, Blmh has a hydrolase activity towards HTL [14,54] (Table 1). Substrate specificity studies of purified human Blmh show that the enzyme exhibits absolute stereospecicity for *L*-HTL, the preferred natural substrate [14]. Methyl esters of *L*-Cys and *L*-Met, but not of other *L*-amino acids, are also hydrolyzed. However, *D*-HTL, *D*-Met methyl ester, γ-thiobutyrolactone, *L*-homoserine lactone are not hydrolyzed by Blmh [14].

HTLase activity of Blmh is significantly reduced in brains from Alzheimer's disease patients compared with unaffected brains [41]. This finding suggests that diminished functional Blmh activity could contribute to the pathology of the disease.

Catabolism of HTL is impaired in *Blmh*$^{-/-}$ mice. For example, *Blmh*$^{-/-}$ mice have elevated brain and kidney HTL, and plasma *N*-Hcy-protein levels compared with wild type *Blmh*$^{+/+}$ littermates [54]. *Blmh*$^{-/-}$ mice are significantly more sensitive to HTL toxicity than their wild type littermates [54].

Biphenyl hydrolase-like protein

Biphenyl hydrolase-like protein (BPHL), also called valacyclovir hydrolase, is a mitochondrial protein highly expressed in human liver and kidney [55,56]. BPHL hydrolyzes and activates the antiviral prodrug esters valacyclovir and valganciclovir, used in the management of herpes simplex, herpes zoster (shingles) and herpes B [57]. First cloned from breast carcinoma cells, BPHL, a member of the alpha/beta hydrolase fold family, is a serine hydrolase distantly related to other members of the serine hydrolase family [55,56]. In mice, deletion of the *Bphl* gene results decreased circulating creatinine levels in males, suggesting a kidney function defect (http://www.informatics.jax.org/allele/allgenoviews/MGI:5548556).

First reports showing that human BPHL has an HTLase activity were published in 2010-2011 [15,16] and confirmed in 2014 [17]. Although BPHL, BLMH, and PON1, hydrolyze HTL, they differ in catalytic efficiencies and exhibit distinct specificities towards non-physiological substrates (Table 1). Catalytic efficiency of BPHL for HTL hydrolysis is higher than that of BLMH or PON1, suggesting a significant HTL- detoxifying role for BPHL *in vivo*, which remains to

Table 1. Substrate specificities of human HTLases.

Substrate	PON1 activity, %	BLMH activity, %	BPHL activity, %
L-Hcy-thiolactone (k_{cat}/K_m)	100 ($10\ M^{-1}s^{-1}$)	100 ($10^3\ M^{-1}s^{-1}$)	100 ($7.7x10^4\ M^{-1}s^{-1}$)
D-Hcy-thiolactone	24	<1	ND
γ-Thiobutyrolactone	545	<1	<0.001
N-Acetyl-*D,L*-HTL	<1	<1	<0.001
L-Hse-lactone	++++	–	+++
L-Met methyl ester	<1	++	30
L-Cys methyl ester	<1	++	++
L-Lys methyl ester	ND	–	–
L-Phe ethyl ester	0	ND	16
Nε-Hcy-aminocaproate	ND	++++	ND
Val(*Nε*-Hcy-Lys)	ND	++++	ND
HcyLeuAla	ND	++++	ND
Bleomycin	ND	500	ND
Paraoxon	330	–	ND
Phenyl acetate	280,000	–	<0.001
Valacyclovir	–	ND	22

be examined in future studies.

HTL clearance by renal excretion

In humans and mice endogenous HTL is also eliminated by urinary excretion [58,59]. HTL concentrations in urine vary from 11 nM to 485 nM and are 100-forld higher than in plasma. Urinary HTL accounts for 2.5% - 28% of urinary tHcy. Relative renal clearance of HTL is 0.2 – 7.0 of creatinine clearance, while clearance of tHcy is only about 0.001 – 0.003 [58]. Efficient urinary elimination of HTL is typical for the waste or toxic products of normal human metabolism.

Calculations based on a normal glomerular filtration rate of 180 L/day and a free plasma Hcy concentrations of 3 μM indicate that 99% of filtered tHcy is reabsorbed [60] in humans. A similar calculation for HTL (0.12 - 2.4 nM in plasma and 286 - 415 nmol/day eliminated in urine) indicates that only 0.4 - 3.8% is reabsorbed and >95% of filtered HTL was excreted [58].

In mice fed with a normal chow diet, urinary HTL is 140 nM [59], similar to urinary HTL value in humans [58]. However, in mice with dietary (high-Met) or genetic (*Cbs*$^{-/-}$) hyperhomocysteinemia urinary HTL increases 25-fold [59] or >50-fold [61]. The distributions of HTL between plasma and urine in mice fed a normal diet and humans are similar: HTL accumulates to much higher levels in urine than in plasma (the ratio urinary/plasma HTL is 37 in mice [59] and 100 in humans [58]). This shows that urinary clearances of HTL in mice and humans are similar, and that in mice, similar to humans [58], >95% of the filtered HTL is excreted with urine. Furthermore, significantly higher urinary/plasma HTL ratios are found in mice fed with a high-Met diet than in the animals fed a normal diet, which suggests that efficiency of urinary HTL clearance increases in hyperhomocysteinemia.

Renal excretion removes a large fraction of HTL [58] that would otherwise cause protein damage by *N*-homocysteinylation. Thus, urinary excretion is an important route of HTL elimination and intact renal function is essential for HTL detoxification in humans and mice.

Implications

Given the role of PON1, BLMH, and BPHL in the detoxification of Hcy-thiolactone, available evidence supports the possibility that elevated HTLase activity might protect against CVD in the general population. Dietary, pharmacological, or genetic interventions to increase or preserve HTLase activity might provide basis for CVD prevention or treatment. Such strategies are feasible with PON1 as shown by studies in which dietary consumption of red wine or its flavonoids quercetin and catechinwas shown to preserve serum PON1 activity in *ApoE*$^{-/-}$ mice [62]. Pomegranate juice, which is rich in flavonoids, causes significant elevations in PON1 activity in humans and reduces the size of atherosclerotic lesions in *ApoE*$^{-/-}$ mice [63]. Further, PON1 overexpression in mice inhibits atherosclerosis development [64]. Identification of determinants of PON1, BLMH, and BPHL activity and subsequent human intervention studies are needed to examine these potential therapeutic implications.

Acknowledgements

Studies from the Author's lab were supported in part by grants 2012/07/B/NZ7/01178, 2013/09/B/NZ5/02794, 2013/11/B/NZ1/00091 from the National Science Center and 12GRNT9420014 from the American Heart Association.

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