Genetic determinants of homocysteine thiolactonase activity in humans: implications for atherosclerosis

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Abstract A metabolite of homocysteine (Hcy), the thioester Hcy thiolactone, damages proteins by modifying their lysine residues which may underlie Hcy-associated cardiovascular disease in humans. A protein component of high density lipoprotein, Hcy thiolactonase (HTase) hydrolyzes thiolactone to Hcy. Thiolactonase is a product of the polymorphic *PON1* gene, also involved in detoxification of organophospates and implicated in cardiovascular disease. Polymorphism in *PON1* affects the detoxifying activity of PON1 in a substrate-dependent manner. However, how *PON1* polymorphism affects HTase activity is unknown. Here we report a strong association between the thiolactonase activity and *PON1* genotype in human populations. High thiolactonase activity was associated with L55 and R192 alleles, more frequent in blacks than in whites. Low thiolactonase activity was associated with M55 and Q192 alleles, more frequent in whites than in blacks. High thiolactonase activity afforded better protection against protein homocysteinylation than low thiolactonase activity. These results suggest that variations in HTase may play a role in Hcy-associated cardiovascular disease.

Key words: Homocysteine thiolactonase; Paraoxonase; Genetic variation; Protein homocysteinylation

1. Introduction

Elevated serum homocysteine (Hcy) is an independent risk factor for cardiovascular disease in humans [1,2]. Although the exact mechanism of Hcy toxicity is unknown, it is believed that Hcy or its metabolite adversely affects vascular endothelium whose damage is a hallmark of atherosclerosis [3]. We have defined a highly specific chemical mechanism by which Hcy can adversely affect cell function [4–8]: Hcy is first converted by methionyl-tRNA synthetase to Hcy thiolactone [9–12]) which then reacts with lysine residues in proteins, damaging their structure and impairing their physiological activities [7]. The extent of Hcy thiolactone synthesis and protein homocysteinylation in human vascular endothelial cells depend on levels of Hcy, Met, folate and high density lipoprotein (HDL) [8], known to be linked to vascular disease [1–3].

Human serum Hcy thiolactonase (HTase), a component of HDL, hydrolyzes Hcy thiolactone to Hcy [13]. HTase also hydrolyzes the organophosphate paraoxon and is identical with paraoxonase [13], a product of the *PON1* gene [14]. Hcy thiolactone is a likely natural substrate of HTase/paraoxonase [13]. Paraoxonase can also act on oxidized phospholipids from low density lipoprotein [20]. Human *PON1* has several genetic polymorphisms [15], two of which result in amino acid substitutions at positions 55 and 192 in PON1 proteins [16,17]. Effects of these polymorphisms on the hydrolysis of organophosphates, aryl esters and nerve gases (sarin, soman) are substrate-dependent. For example, the R192 and Q192 isoforms exhibit high and low activity, respectively, with paraoxon, but low and high activity, respectively, with sarin; and do not affect the activity toward phenyl acetate [18]. Paraoxonase protects against atherosclerosis in a mouse model [19] and *PON1* polymorphisms are implicated in human cardiovascular disease [20].

However, how these polymorphisms affect HTase activity is unknown. As a first step towards assessing its role in Hcy-associated vascular disease, we examined HTase activities and genotypes in human populations. We report that high HTase activity was associated with L55 and R192 alleles, more prevalent in blacks than in whites, whereas low HTase activity was associated with M55 and Q192 alleles, more prevalent in whites than in blacks. We also found that the high activity form of HTase affords a better protection against protein homocysteinylation than the low activity form.

2. Materials and methods

2.1. Human subjects

The subjects (Table 1) were young healthy recruits from a longitudinal study of blood pressure regulation [21]. They were admitted to an in-patient research facility (General Clinical Research Center) the night before. The young age avoids potential confounding effects associated with adult subjects. Fasting blood samples were obtained the following morning at 07.00 h while subjects were still recumbent. Serum [13] for enzymatic assays and DNA [22] for genotyping were prepared from the blood obtained from the subjects via venipuncture with informed consent.

2.2. Enzyme assays

Hydrolysis rates of 1 mM [^{35}S]Hcy thiolactone and 1 mM paraoxon were determined as described [13]. These rates were corrected for non-enzymatic hydrolysis of substrates which was determined by inactivating HTase with 5 mM EDTA/2 mM D-penicillamine [13]. 1 U of HTase is defined as the amount of enzyme capable of hydrolyzing 100 nmol [^{35}S]Hcy thiolactone to [^{35}S]Hcy within 30 min at 37°C. 1 U of paraoxonase is defined as the amount of enzyme that will

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Table 1
Description of subjects

	Blacks ($n=33$)	Whites ($n=34$)	P-value
Gender (M/F)	14/19	14/20	1.000
Age (years)	15.8 ± 2.9	18.0 ± 3.6	0.0077
HTase (U)	13.7 ± 4.1	9.9 ± 3.5	0.0001
Paraoxonase (U)	243.2 ± 108.0	143.8 ± 93.3	0.0001

Values are presented as mean ± S.D. Fisher's exact test was used for gender and the two-sample t-test for the remainder.

hydrolyze paraoxon to p-nitrophenol at a rate of $0.001A_{412}$ U/ml of p-nitrophenol per 10 min at 25°C.

2.3. Genotyping

Genotypes of the 55 and 192 *PON1* alleles [17] were determined as described. DNA was prepared from peripheral white blood cells using a standard procedure [21]. Polymerase chain reaction amplifications were carried out using the following DNA primers: forward 192 primer 5′-TATTGTTGCTGTGGGACCTGAG-3′, the reverse 192 primer 5′-CACGCTAAACCCAAATACATCTC-3′, the forward 55 primer 5′-GAAGAGTGATGTATAGCCCCAG-3′ and the reverse 55 primer 5′-TTTAATCCAGAGCTAATGAAAGCC-3′. The amplification reactions (40 cycles) used REDTaqDNA Polymerase (Sigma). For alleles 192 each cycle included 60 s at 94°C, 30 s at 65°C, and 60 s at 72°C. For alleles 55 each cycle included 45 s at 94°C, 45 s at 61°C, and 45 s at 72°C. Reactions were denatured with 0.4 M NaOH, 25 mM EDTA at 94°C for 5 min. Nytran membranes were blotted and then heated at 80°C for 1 h under vacuum. Allele-specific hybridization was performed using the following end-labeled probes: Q192-specific 5′-CCTACTTACA**A**TCCTGGG-3′, R192-specific 5′-CCTACTTAC**G**ATCCTGGG-3′, M55-specific 5′-CTGAAGACA**T**GGAGATA-3′ and L55-specific 5′-CTGAAGAC**T**TGGAGATA-3′. Membranes were pre-hybridized for 1 h at 42°C, then hybridized overnight at 55°C for alleles 192 or at 43°C for alleles 55. Blotted probes were washed for 5 min at room temperature in 2×SSC and 0.5% SDS (1×SSC for Q192) and for 5 min at 40°C.

2.4. Statistics

The two-sample t-test was used to compare age and enzyme activity levels between the black and white cohorts and to examine for gender differences. The Cochran–Mantel–Haenszel test of association was used to examine for linkage disequilibrium between the 55 and 192 *PON1* alleles. The exact Cochran–Armitage trend test [24] was used to examine for racial differences in the distributions of the 55 and 192 *PON1* alleles. All analysis of HTase and paraoxonase activity levels was done on the logarithmic scale to equalize variances. Three-way analysis of variance was used to examine for genotype and gender effects. Both genotype effects were modeled as three-level categorical factors. Adjusted means and their standard errors (S.E.M.) are reported in Table 3. In Table 3, we have included the exponentiated adjusted means to bring the results back to the original scale.

3. Results

3.1. Frequencies of HTase/paraoxonase polymorphisms

A description of the study population is presented in Table 1. The genotype distributions of HTase/paraoxonase polymorphisms were determined in 67 young subjects. The frequencies of the 55 *PON1* polymorphism were 3.0% MM, 30.3% ML, 66.7% LL in blacks and 14.7% MM, 38.2% ML, 47.1% LL in whites ($P=0.06$). The frequencies of the 192 *PON1* polymorphism were 12.1% QQ, 48.5% QR, 39.4% RR in blacks and 44.1% QQ, 52.9% QR, 2.9% RR in whites ($P<0.0001$). The 55 and 192 *PON1* alleles were in the Hardy–Weinberg equilibrium [23] in each race group ($P=0.02$). There was a marginal evidence for linkage disequilibrium of the 55 with the 192 allele in the whites ($P=0.0515$) but none in the blacks ($P=0.543$).

The frequencies of the 55 and 192 genotypes in blacks were not studied before. The frequencies of the 192QQ and 192RR genotypes in blacks reported here are similar to the frequencies of associated phenotypes in other black populations [16]. The frequencies of the 55 and 192 *PON1* alleles in whites reported here are similar to those observed in other white populations [16,17]. Altogether, frequencies of the L55 and R192 alleles in the black population (0.82 L55 and 0.64 R192) were higher than in the white population (0.64 L55 and 0.29 R192). This results in 67 and 39% of black individuals being homozygous for the L55 and R192 forms, respectively, compared with 47 and 3%, respectively, of white individuals. Conversely, only 3 and 12% of blacks were homozygous for the M55 and Q192 forms, respectively, compared with 15 and 44% of whites (Table 2). The differences in allele distribution between blacks and whites were highly significant for the 192 alleles ($P<0.0001$) and marginally significant for the 55 alleles ($P=0.06$).

3.2. Variations in HTase activity

We also determined the rates of Hcy thiolactone hydrolysis in relation to the rates of paraoxon hydrolysis by HTase/paraoxonase in sera from black and white young volunteers. There was about five-fold variation in serum HTase activity in these populations, which paralleled the variation in serum paraoxonase activity (Fig. 1). Plots of the HTase activity against the paraoxonase activity showed a positive correlation between the two activities ($r=0.89$), similar for blacks (Fig. 1A) and whites (Fig. 1B). The HTase versus paraoxonase plots are uniphasic (Fig. 1), in contrast to the triphasic plots of chloropyrifos-oxonase versus paraoxonase, arylesterase versus paraoxonase, diazooxonase versus paraoxonase, somanase versus paraoxonase, and sarinase versus paraoxonase [18]. It is unclear whether the uniphasic nature of the HTase versus paraoxonase plot is specific for the natural substrate Hcy thiolactone, or reflects population differences between the two studies.

Table 2
Racial distributions of the 55 and 192 alleles of *PON1*

Population	55MM	55ML	55LL	192QQ	192QR	192RR
Black	1 (3.0%)	10 (30.3%)	22 (66.7%)	4 (12.1%)	16 (48.5%)	13 (39.4%)
White	5 (14.7%)	13 (38.2%)	16 (47.1%)	15 (44.1%)	18 (52.9%)	1 (2.9%)

The distributions of 55 and 192 alleles showed a marginally significant ($P=0.06$) and significant ($P<0.0001$) difference, respectively, between blacks and whites. Values listed in parentheses are percentages within a racial group.

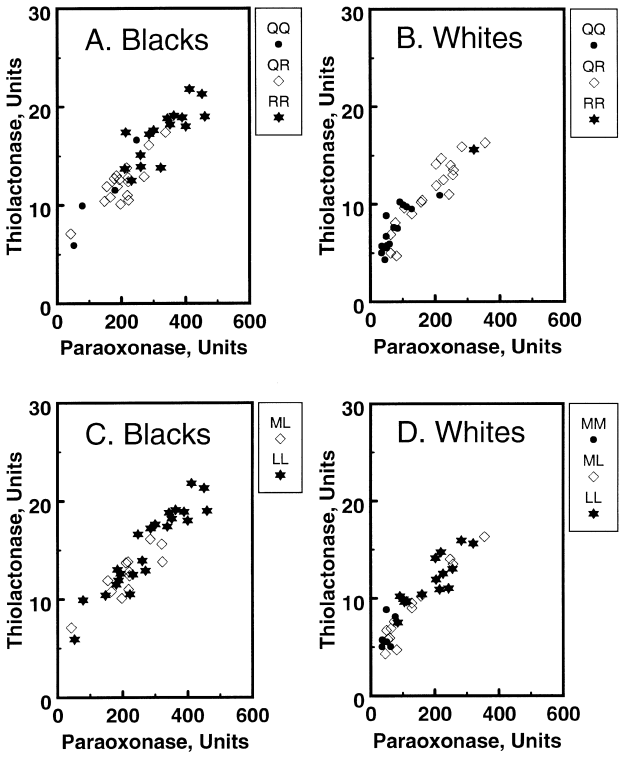

Fig. 1. Population distribution plots of HTase versus paraoxonase activities by race and genotype. A, C: Blacks. B, D: Whites. Polymorphisms of the 192 allele (A, B) and the 55 allele (C, D) are indicated.

3.3. Associations between HTase activity and PON1 genotype

As shown in Table 3, high HTase activity, was associated with the L55 ($P = 0.0027$ and 0.0056 for blacks and whites, respectively) and R192 alleles ($P = 0.0005$ and 0.0127 for blacks and whites, respectively). Low HTase activity was associated with the M55 and Q192 alleles in both races. Blacks were more likely than whites to have the high activity L55 and R192 alleles ($P = 0.0001$). Conversely, whites were more likely than blacks to have the low activity M55 and Q192 alleles (Table 3). Because the differences in HTase activity between individuals were rather large (up to five-fold), our analysis of the associations between HTase activity and genotypes reached statistical significance with a relatively small number of subjects (Tables 1–3). For none of the models was gender a significant predictor ($P > 0.37$).

Similar associations were also observed between the paraoxonase activity and *PON1* genotypes in blacks and whites (not shown), consistent with other studies [16]. Thus, although effects of *PON1* genotypes on activity are substrate-dependent in most cases [18], the genotype–activity relationships are identical for HTase and paraoxonase, explaining a positive correlation between the two activities (Fig. 1).

3.4. HTase accelerates Hcy thiolactone turnover and minimizes protein homocysteinylation

To assess a possible significance of the variation in HTase levels in humans, the turnover of Hcy thiolactone was studied in sera from the 55LL/192RR (high activity) and 55MM/192QQ (low activity) donors. As shown in Fig. 2A, in sera from the high activity HTase donors, Hcy thiolactone (half life of 0.5 h) disappeared about twice as fast as in sera from the low activity HTase donors (half life of 1.0 h). When HTase activity was inactivated in either serum by the supplementation with EDTA and D-penicillamine [13], Hcy thiolactone disappeared more slowly, with a half life of 1.5 h (Fig. 2A). The disappearance of thiolactone was accompanied by a progressive accumulation of Hcy (Fig. 2B) and Hcy-protein (Fig. 2C) in relative amounts depending on the level of HTase

Table 3
Adjusted means (±S.E.M.) of the logarithm of HTase activity levels for different *PON1* alleles

PON1 Allele	Blacks			Whites		
	adjusted mean	adjusted original scale	*P*-value	adjusted mean	adjusted original scale	*P*-value
55MM	1.63 ($n = 1$)	5.12	0.0027	1.96 ± 0.13 ($n = 5$)	7.12	0.0056
55ML	2.56 ± 0.08 ($n = 10$)	12.94		2.10 ± 0.08 ($n = 13$)	8.16	
55LL	2.61 ± 0.05 ($n = 22$)	13.64		2.41 ± 0.07 ($n = 16$)	11.16	
192QQ	2.27 ± 0.12 ($n = 4$)	9.69	0.0005	2.05 ± 0.07 ($n = 15$)	7.74	0.0127
192QR	2.45 ± 0.06 ($n = 16$)	11.57		2.36 ± 0.07 ($n = 18$)	10.56	
192RR	2.81 ± 0.07 ($n = 13$)	16.53		2.56 ± 0.2 ($n = 1$)	12.95	

Adjusted means were calculated using the log-transformed data. These were exponentiated to obtain adjusted values on the original scale. The *P*-values are adjusted for gender and the other genotype.

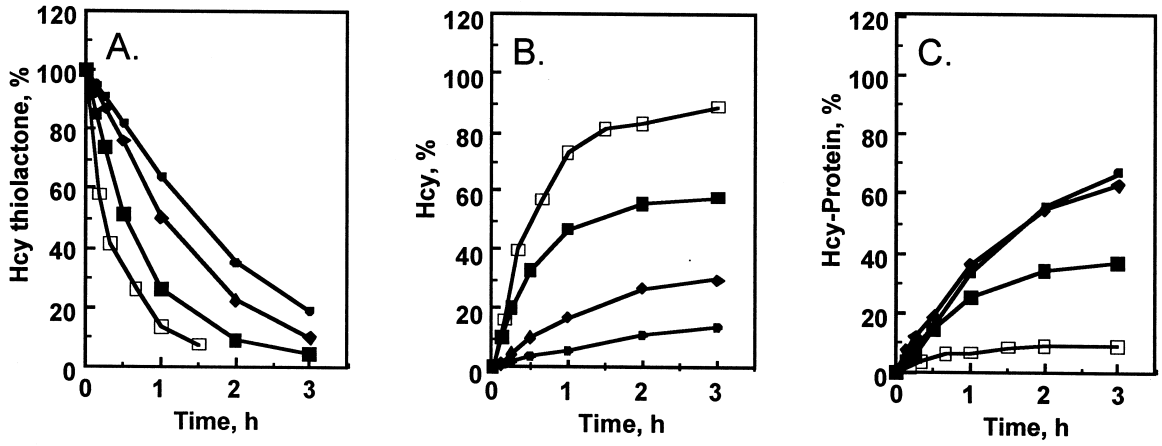

Fig. 2. Turnover of Hcy thiolactone in serum. Serum was spiked with 3.5 μM [^{35}S]Hcy thiolactone (20 000 Ci/mol) and incubated at 37°C for up to 3 h. At indicated time intervals, aliquots of ^{35}S-labeled serum were diluted four-fold with 5 mM dithiothreitol and Hcy thiolactone and the products of its turnover, Hcy and Hcy-protein, were separated by thin layer chromatography on cellulose plates (Analtech) [13]. Kinetics of disappearance of Hcy thiolactone (A), formation of Hcy (B) and Hcy-protein (C) in human serum from high HTase activity donors (genotype LL55/RR192; ■), low HTase activity donors (genotype MM55/QQ192; ♦), rabbit serum (□), and in human sera in which HTase was inactivated by supplementation with 5 mM EDTA and 2 mM D-penicillamine (●) are shown.

activity in serum. More Hcy and less Hcy-protein accumulated in sera from high HTase activity donors than in sera from low HTase activity donors. The high activity form of HTase afforded much better protection against protein homocysteinylation than the low activity form (Fig. 2C).

For comparison, we have also analyzed Hcy thiolactone turnover in rabbit serum, known to possess the highest levels of HTase activity among vertebrates [6,7,13]. Hcy thiolactone (half life of 15 min) disappeared twice as fast in rabbit serum than in human serum from the 55LL/192RR HTase donors (half life of 30 min) (Fig. 2A). More Hcy and less Hcy-protein accumulated in rabbit serum than in human serum from the 55LL/192RR HTase donor. Thus, rabbit serum HTase affords much better protection against protein homocysteinylation than human serum HTase.

4. Discussion

This work shows that (1) there is an important variation in HTase activity in human beings; (2) high HTase activity is associated with L55 and R192 *PON1* alleles, low HTase activity is associated with M55 and Q192 *PON1* alleles; (3) there are large differences in the genotype frequencies in black and white human populations, explaining the differences in HTase activity levels; (4) the HTase activity correlates with the paraoxonase activity in both populations and (5) high activity forms of HTase afford better protection against protein homocysteinylation than low activity forms. These data suggest that HTase activity/genotype may have relevance to a risk for Hcy-linked cardiovascular disease in human populations.

The mechanisms underlying the association between Hcy and cardiovascular diseases are unclear. One possible mechanism involves metabolic conversion of Hcy to Hcy thiolactone and protein homocysteinylation by Hcy thiolactone, which results in protein damage [7]. The presence in human beings of a HDL-associated HTase which hydrolyzes Hcy thiolactone [13] supports this mechanism. The variation in HTase activity and the association of high activity with specific *PON1* alleles (L55 and R192) in human populations, described in this study, suggest that HTase activity and/or genotype might be important factors in Hcy-associated vascular disease. However, because the distributions of HTase activity within genotypes overlap (Fig. 1), HTase activity might be a better predictor than the *PON1* genotype. High HTase activity affords better protection against protein homocysteinylation than low HTase activity (Fig. 2). Although Hcy-protein is present in human serum [5] it is unknown how the levels of Hcy-protein correlate with HTase levels.

Higher HTase activity and a greater frequency of high activity *PON1* alleles in blacks than in whites are striking, but correlate well with higher HDL levels in blacks compared with whites. Thus, blacks, in addition to more favorable lipid profiles [25], appear to have also more favorable Hcy thiolactone metabolism. However, associations between Hcy and myocardial infarction are similar among blacks and whites [2], which suggests that environmental factors may be removing any natural advantage that blacks may have.

Because paraoxonase may remove lipid peroxidation products, numerous studies examined a possible relationship between *PON1* polymorphisms and coronary heart disease [20]. The R192 allele was associated with an increased risk for cardiovascular disease in some populations at high risk [15], but not in populations at large [26]. It has been suggested that paraoxonase *activity* may be a better predictor for cardiovascular disease or organophosphate susceptibility than the genotype alone [27].

Our present data lead us to hypothesize that low HTase activity may be a risk factor in populations with elevated Hcy levels. No clinical studies have yet examined relationships between HTase activity and cardiovascular disease. However, two recent clinical studies support our hypothesis that the low activity HTase/paraoxonase alleles (Q192 and M55), combined with elevated Hcy, may predispose humans to cardiovascular disease. In one clinical trial [28], the Q192 allele of *PON1* was associated with the extent of coronary artery disease in human subjects homozygous for the methylenetetrahydrofolate reductase T677 genotype (i.e. having elevated Hcy levels). In another study [29], Finnish subjects homozygous for the low activity M55 paraoxonase allele were about three

times more likely than the high activity L55 homozygotes to suffer a myocardial infarction. Although not examined in that study [29], Hcy levels in Finns are known to be the highest among populations in industrialized countries, as is the prevalence of cardiovascular disease mortality [30]. These observations underline the importance of examining the variability of HTase and protein homocysteinylation in future studies of associations between Hcy and vascular diseases in human populations.

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