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Metabolism and Neurotoxicity of Homocysteine Thiolactone in Mice: Evidence for a Protective Role of Paraoxonase 1

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Abstract

Homocysteine (Hcy)-thiolactone is toxic, induces epileptic seizures in rodents, and has been implicated in Alzheimer's disease. Paraoxonase 1 (Pon1), a component of high-density lipoprotein, hydrolyzes Hcy-thiolactone *in vitro*. Whether this reflects a physiological function and whether Pon1 can protect against Hcy-thiolactone toxicity was unknown. Here we show that Hcy-thiolactone was elevated in brains of *Pon1*$^{-/-}$ mice (1.5-fold, $p = 0.047$) and that *Pon1*$^{-/-}$ mice excrete more Hcy-thiolactone than wild type animals (2.4-fold, $p = 0.047$). The frequency of seizures induced by intraperitoneal injections of *L*-Hcy-thiolactone was significantly higher in *Pon1*$^{-/-}$ mice compared with wild type animals (52.8% versus 29.5%, $p = 0.042$); the latency of seizures was lower in *Pon1*$^{-/-}$ mice than in wild type animals (31.8 min versus 41.2 min, $p = 0.019$). Using the *Pon1* null mice, we provide the first direct evidence that a specific Hcy metabolite, Hcy-thiolactone, rather than Hcy itself is neurotoxic *in vivo*. Our findings indicate that Pon1 protects mice against Hcy-thiolactone neurotoxicity by hydrolyzing it in the brain, and suggest a mechanism by which Pon1 can protect against neurodegeneration associated with hyperhomocysteinemia and Alzheimer's disease.

Keywords

Alzheimer's disease; homocysteine thiolactone; neurotoxicity; Pon 1

INTRODUCTION

Homocysteine (Hcy) arises from the metabolism of the essential dietary protein amino acid methionine (Met). Hcy levels are regulated by remethylation to Met, catalyzed by Met synthase (with methyltetrahydrofolate cofactor generated by methylenetetrahydrofolate reductase) and betaine-Hcy methyltransferase, as well as by transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β-synthase. Genetic or nutritional

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deficiencies in these pathways cause hyperhomocysteinemia, homocystinuria, and lead to heart and brain pathologies [1].

Hcy is also metabolized by methionyl-tRNA synthetase [2] to a chemically reactive thioester, Hcy-thiolactone, which spontaneously *N*-homocysteinylates protein lysine residues generating *N*-Hcy-protein [3]. The accumulation of Hcy-thiolactone [4] and *N*-Hcy-protein [5, 6] greatly increases in genetic or nutritional hyperhomocysteinemia. *N*-Homocysteinylation causes protein damage [3, 7] by a thyil radical mechanism [8], generates amyloid-like structures [9], and is linked to atherosclerosis [10] and Alzheimer's disease (AD) [11]. Human clinical studies show that elevated plasma Hcy is a risk factor for dementia and AD and that Hcy lowering by B-vitamin treatment slows the rate of brain atrophy [12].

Serum paraoxonase 1 (Pon1) is synthesized exclusively in the liver and attached to high-density lipoproteins (HDL) in the blood, but recent studies demonstrate that Pon1 is also present in the brain [13]. Pon1 protects against high-fat diet-induced atherosclerosis in mice [14] and humans [15]. *N*-homocysteinylated forms of HDL [16] and ApoA1 [17] have been detected in human plasma *in vivo*. *In vitro*, *N*-homocysteinylation of HDL and Pon1 causes a loss of function [18]. In humans, Pon1 is implicated in 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 [12].

HDL and purified Pon1 protein have the ability to hydrolyze Hcy-thiolactone *in vitro* [24], but whether this reflects a physiological function was unknown. Intraperitoneal (i.p.) injections of Hcy-thiolactone are acutely neurotoxic and have been extensively used to define mechanism of seizures in mice [25, 26] and rats [27, 28]. To gain insight into a role of Pon1 in Hcy-thiolactone metabolism and brain disease, we exploited the *Pon1*$^{-/-}$ mouse [14] and the i.p., Hcy-thiolactone injection models.

MATERIALS AND METHODS

Mice and diet

Knockout *Pon1*$^{-/-}$ mice on the C57BL/6J background [14] and wild type *Pon1*$^{+/+}$ littermates were maintained on a rodent chow (LabDiet 5010, Purina Mills Intl, St. Louis, MO). To induce hyperhomocysteinemia, 4-weeks-old mice were provided 1% Met in drinking water for 8 weeks [29]. Animal procedures were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School.

Mouse plasma and urine

The blood was collected by cheek vein puncture into EDTA tubes and chilled on ice. Plasma was separated and stored at −80°C. Urine was collected at about 2 h intervals for a period of 24-h, each portion chilled on ice and stored at −80°C.

Hcy-thiolactone and Hcy turnover

L-Hcy-thiolactone dissolved in PBS was injected i.p., into 4-12-week-old mice (40-600 nmol/g body weight). The mice were bled 5, 10, 20, and 30 min (for Hcy-thiolactone assays) or 10, 20, 30, 45, 60, 75, 90 min (for Hcy assays) after injection. EDTA-Plasma samples were stored at −80°C before assay.

Hcy-thiolactone toxicity

L-Hcy-thiolactone dissolved in PBS was injected i.p., into 4-5 week-old mice (3,700 nmol/g body weight). Mice were placed on the top of a plastic cage and observed for behavioral

manifestations for 90 min. This was assessed by the incidence and latency of seizures and death as previously described [25, 26].

Hcy-thiolactone and total Hcy assays

Hcy-thiolactone and total Hcy were quantified by HPLC-based methods using post-column derivatization with *o*-phthaldialdehyde and fluorescence detection as previously described [30, 31].

RESULTS

Inactivation of Pon1 elevates brain Hcy-thiolactone

We found that Hcy-thiolactone levels were significantly elevated in brains of *Pon1*$^{-/-}$ mice, in comparison with *Pon1*$^{+/+}$ animals (Table 1). However, Hcy-thiolactone levels in heart, kidney, liver, lung, spleen, and plasma were not significantly affected by the inactivation of *Pon1* gene (Table 1). The elevation of Hcy-thiolactone could be due to effects of *Pon1* on Hcy levels. To test this possibility, we assayed brain total Hcy. We found that brain tHcy levels were similar in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice, 46.4 ± 13.4 and 59.7 ± 18.3 pmol/mg tissue, respectively. Thus, the increase in Hcy-thiolactone levels observed in *Pon1*$^{-/-}$ mice is caused by inactivation of the *Pon1* gene, and not by its effects on Hcy metabolism.

Pon1$^{-/-}$ mice excrete more Hcy-tiolactone than wild type animals

Most of Hcy-thiolactone produced in the human [31] or mouse [4] body is excreted in urine. To facilitate quantification of urinary Hcy-thiolactone, we induced hyperhomocysteinemia by providing *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice with 1% Met in drinking water for 8 weeks. The consumption of Met-supplemented water (3.1 mL/mouse) was not affected by *Pon1* genotype. Plasma tHcy levels in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice fed with Met-supplemented drinking water were elevated 5.6- and 10.4-fold (to 48 ± 16 μM, and 77 45 μM, respectively, from a basal level of 8.5 ± 1.9 μM and 7.4 2.2 ± μM, respectively), in mice that ± drank non-supplemented water. 24-h urine was collected for Hcy-thiolactone quantification from *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice. We found that *Pon1*$^{-/-}$ mice excreted 2.4-fold more Hcy-thiolactone in 24-h urine than *Pon1*$^{+/+}$ animals (Table 2). In contrast, urinary tHcy levels were similar in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice. Unexpectedly, we found that *Pon1*$^{-/-}$ mice generated more urine than wild type animals, suggesting that Pon1 might be involved in the regulation of kidney function, which warrants investigation in future studies.

Turnover of plasma Hcy-thiolactone in vivo

To determine how *Pon1* affects its turnover *in vivo*, *L*-Hcy-thiolactone was injected i.p. into *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice, and Hcy-thiolactone kinetics in plasma were monitored. We used a non-toxic dose of 600 nmol *L*-Hcy-thiolactone/g body weight in most experiments, but similar results were obtained with doses as low as 40 nmol/g body weight. The highest Hcy-thiolactone levels were observed at 5 min post-injection and were not affected by *Pon1* status (Fig. 1A). This shows that in *Pon1*$^{-/-}$ mice Hcy-thiolactone metabolism is not impaired during its transit from the intraperitoneal cavity to the bloodstream and suggests that *Pon1* has a negligible contribution to Hcy-thiolactone turnover in membranes surrounding the intraperitoneal cavity.

Because of its exceptionally low pK = 6.7 [32], Hcy-thiolactone is mostly neutral under physiological conditions and freely diffuses through cell membranes and is found in extracellular media [33, 34]. Assuming that the i.p.,-injected Hcy-thiolactone distributes uniformly throughout the body and that blood constitutes 8% of the mouse body weight, plasma Hcy-thiolactone extrapolated to time zero (HTL^{0}, Table 3) represents 6.6% or 5.9% of the dose injected into *Pon1*$^{-/-}$ or *Pon1*$^{+/+}$ mice, respectively.

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Hcy-thiolactone gradually decreased with exponential kinetics, approaching basal level at 30 min post-injection (Fig. 1A). Plasma Hcy-thiolactone half-life was similar in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice, 5.9 ± 1.2 min and 5.0 ± 0.9 min, respectively (Table 3), suggesting that *Pon1* does not significantly contribute to Hcy-thiolactone clearance from the blood.

Half-lives of Hcy-thiolactone in serum from *Pon1*$^{+/+}$ and *Pon1*$^{-/-}$ mice were 73 min and >1000 min, respectively, >10-fold longer than the *in vivo* value of about 5 min (Table 3). These values suggest that *Pon1* contributes at most 10% to Hcy-thiolactone clearance from the mouse blood *in vivo*.

Plasma total Hcy kinetics in vivo

In *Pon1*$^{+/+}$ mice i.p.,-injected with *L*-Hcy-thiolactone (600 nmol/g body weight), plasma total Hcy increased to 524 ± 136 μM (extrapolated level at time zero; Fig. 1B, Table 3) from a basal level of 7.4 ± 2.2 μM. This shows that Hcy-thiolactone was metabolized to Hcy. In *Pon1*$^{-/-}$ mice injected with identical dose of *L*-Hcy-thiolactone, plasma total Hcy increased to 256 ± 23 μM (Table 3). The lower post-injection total Hcy levels, suggest that excess Hcy is metabolized (to Met and/or Cys) faster in *Pon1*$^{-/-}$ than in *Pon1*$^{+/+}$ mice. Post-injection plasma total Hcy gradually declined with exponential kinetics and similar half-lives in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice, 27.1 ± 6.3 min and 26.2 2.6 min, respectively (Table 3). Overall, the clearance ± of plasma total Hcy was about 5-times slower than the clearance of plasma Hcy-thiolactone.

Pon1 protects against Hcy-thiolactone neurooxicity

L-Hcy-thiolactone is known to be toxic to rodents [25]. For example, in C3H mice, LD50 and LD10 are 2,540 and 2,390 nmol Hcy-thiolactone/g body weight, respectively [26]. We found that i.p., injections of *L*-Hcy-thiolactone at a dose of 3,700 nmol/g body weight induced seizures and death in 29.5% and 2.3% of *Pon1*$^{+/+}$ C57BL/6J mice (Table 4). Doses from 40 to 2,850 nmol/g mouse body weight were nontoxic.

Following *L*-Hcy-thiolactone injection (3,700 nmol/g body weight), essentially all mice became somnolescent at 5-10 min post-injection. Convulsions, characterized by spontaneous tonic-clonic, grand-mal seizures (Kangaroo position, extension of fore and hind limbs and tail, status epilepticus), and running fits occurred within 50 min. The incidence of seizures significantly increased in *Pon1*$^{-/-}$ mice, to 52.8% ($p = 0.042$, Table 4). Seizure latency (i.e., time to first seizure) was significantly shorter for *Pon1*$^{-/-}$ mice compared with *Pon1*$^{+/+}$ animals (31.8 min versus 41.2 min, $p = 0.019$). Only one *Pon1*$^{+/+}$ mouse, out of 44 (2.3%), died (at 61 min) after *L*-Hcy-thiolactone injection. As also shown in Table 4, the incidence of death increased in *Pon1*$^{-/-}$ mice (to 8.3%) but it was not significantly different from the incidence of death in *Pon1*$^{+/+}$ mice (2.3%), most likely due to small number of animals. Because both *Pon1*$^{+/+}$ and *Pon1*$^{-/-}$ mice are on identical C57BL/6J background, these differences are caused by the *Pon1* gene inactivation and indicate that *Pon1* protects against the toxicity caused by Hcy-thiolactone.

We assayed brain Hcy-thiolactone, *N*-Hcy-protein, and total Hcy 90 min post injection. We found that Hcy-thiolactone and *N*-Hcy-protein levels were higher in *Pon1*$^{-/-}$ than in *Pon1*$^{+/+}$ mice (3.4 1.1 ± 1.5 versus ± ± 0.1 pmol/mg brain, $p = 0.029$, and 48.2 7.7 versus 32.4 ± 6.4 pmol/mg brain, $p = 0.010$, respectively). The post-injection brain total Hcy levels in *Pon1*$^{-/-}$ and *Pon1*$^{+/+}$ mice were not significantly different (425 ± 99 μM versus 634 ± 214 pmol/mg brain).

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DISCUSSION

The physiological function of *Pon1* and its role in brain disease are not fully understood. The present work using the *Pon1* knockout mice provides the first direct evidence that 1) *Pon1* metabolizes L-Hcy-thiolactone to Hcy *in vivo*, and 2) that a specific Hcy metabolite, Hcy-thiolactone, rather than Hcy itself is neurotoxic in mice. These findings suggest a mechanism by which *Pon1* can protect against neurodegeneration associated with hyperhomocysteinemia and AD.

Our data also indicate that, although Hcy-thiolactone is present in most mouse organs examined, *Pon1* significantly contributes to Hcy-thiolactone metabolism mainly in the brain, and that the brain appears to be a major source of urinary Hcy-thiolactone excreted by $Pon1^{-/-}$ mice. This is consistent with recent data showing that *Pon1* is present in the brain [13]. However, the relatively modest and brain-specific increases in Hcy-thiolactone levels in $Pon1^{-/-}$ mice suggest that, in addition to *Pon1*, other enzyme(s) or mechanisms, which remain to be identified, contribute to Hcy-thiolactone turnover in mice. This conclusion is supported by our present findings showing that Hcy-thiolactone is cleared *in vivo* from the mouse blood 15-times faster ($t_{0.5}$ = 5 min) than it is hydrolyzed by *Pon1 in vitro* in serum ($t_{0.5}$ = 73 min), and that the *Pon1* gene inactivation does not significantly affect the *in vivo* Hcy-thiolactone clearance ($t_{0.5}$ = 5.9 min; Table 3).

Acute injections of Hcy-thiolactone are known to cause epileptic seizures in mice [26] and rats [25]. An underlying mechanism may involve the inhibition of Na^+/K^+-ATPase, critical for normal brain function, by Hcy-thiolactone, which would contribute to the seizures. Indeed, the Na^+/K^+-ATPase activity was reported to be greatly diminished by acute injections of Hcy-thiolactone, but not Hcy [28]. Our present data, showing that the inactivation of *Pon1* gene increases the incidence and decreases the latency of seizures, provide the first genetic evidence that *Pon1* protects against Hcy-thiolactone toxicity in mice.

Genetic hyperhomocysteinemia causes neurological abnormalities in humans, manifested by seizures and mental retardation [1]. In a general population, elevated Hcy is a risk factor for dementia and AD [11]. However, it was not known whether Hcy itself or any of its metabolites is responsible for the Hcy-associated neurotoxicity. Using a mouse model with a genetic deficiency in Hcy-thiolactone disposition, $Pon1^{-/-}$, allowed us to examine the role of *Pon1* in the brain pathology caused by acute hyperhomocysteinemia. Findings of the present work, showing that the *Pon1* gene inactivation increases the incidence and decreases the latency of seizures induced by i.p., injections of Hcy-thiolactone in mice, provide the first evidence that Hcy-thiolactone is neurotoxic *in vivo*. Elevated post-injection levels of *N*-Hcy-protein in the brain suggest that the mechanism of Hcy-thiolactone-induced neurotoxicity involves *N*-homocysteinylation of brain proteins. Although in our experimental model Hcy was also generated, post-injection Hcy levels are not significantly different in $Pon1^{-/-}$ and $Pon1^{+/+}$ mice, and do not explain the observed toxicity. Thus, in this model neurotoxicity can be assigned to Hcy-thiolactone, but not to Hcy.

It should be noted that the concentrations of Hcy-thiolactone used in our acute injection experiments greatly exceed physiological concentrations. Such high concentrations, required due to a very efficient metabolism of Hcy-thiolactone in the mouse, caused extreme neurological manifestations within 30-60 minutes. Although much lower Hcy-thiolactone concentrations occur in pathological hyperhomocysteinemia [4], it is likely that under chronic exposure even small amounts of damage caused by Hcy-thiolactone can accumulate to significant levels over extended period of time that is usually required for the development of brain damage, such as observed in AD [12]. Consistent with this scenario is

a recent finding showing that the reduced Hcy-thiolactonase activity is linked to the pathology of AD [11].

In conclusion, using the *Pon1*$^{-/-}$ mice, we provide the first direct evidence that Hcy-thiolactone, rather than Hcy itself is neurotoxic *in vivo*. Our findings indicate that *Pon1* protects mice against the neurotoxicity of Hcy-thiolactone by hydrolyzing it in the brain, suggest a mechanism by which *Pon1* can protect against neurodegeneration associated with hyperhomocysteinemia and AD, and provide basis for future studies of *Pon1*'s role in AD.

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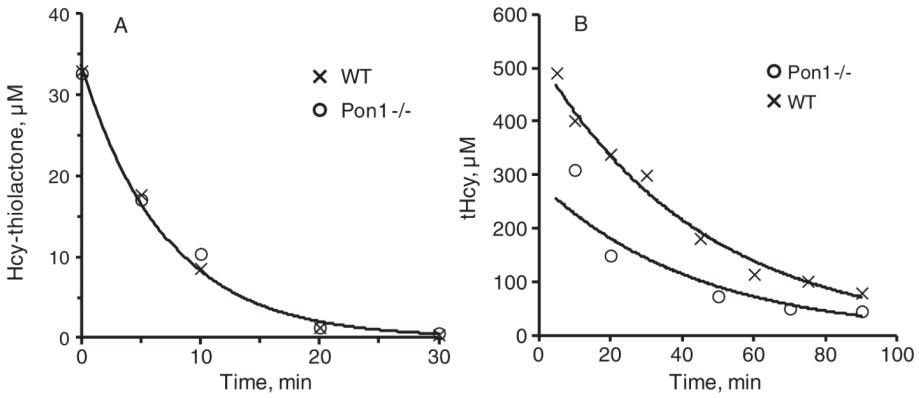

Fig. 1.

Kinetics of plasma Hcy-thiolactone and total Hcy in mice. Representative kinetics of Hcy-thiolactone (A) and tHcy (B) turnover obtained for individual *Pon1*$^{-/-}$ (○) and *Pon1*$^{+/+}$(x) mice injected intraperitoneally with 600 nmol *L*-Hcy-thiolactone/g body weight are shown. Data points were fitted to the exponential equation $[A^t] = [A^0]\cdot e^{-k\cdot t}$, where k is a first order rate constant, $[A^t]$ and $[A^0]$ are measured concentrations at time t and extrapolated concentrations at time zero, respectively.

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Table 1

Tissue levels of Hcy-thiolactone in *Pon1*$^{-/-}$ and wild type mice

Genotype (n)	Hcy-thiolactone, pmol/mg tissue						
	Brain	**Heart**	**Kidney**	**Liver**	**Lung**	**Spleen**	**Plasma**
Pon1$^{-/-}$ (4)	0.51 ± 0.13*	0.11 ± 0.03	0.40 ± 0.01		0.13 ± 0.02	0.09 ± 0.13	0.076 ± 0.047
Wild type (6)	0.33 ± 0.15	0.10 ± 0.02	0.50 ± 0.27	<0.05	0.22 ± 0.13	0.12 ± 0.14	0.112 ± 0.078

*Significantly increased versus wild type: *T*-test: $p = 0.047$.

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Table 2

Urinary Hcy-thiolactone and total Hcy output in *Pon1*$^{-/-}$ and wild type mice. The mice were fed with a 1% Met-supplemented drinking water for 8 weeks. Consumption of Met-supplemented water (3.1 mL/ mouse) was not affected by the genotype

Genotype (*n*)	Urinary Hcy-thiolactone, nmol/24 h	Urinary tHcy, nmol/24 h	Urine volume, mL/24 h	Mouse body weight, *g*
Pon1$^{-/-}$ (4)	3.2 ± 0.5 *	1004 ± 207	1.24 ± 0.19 *	23.4 ± 1.4
Wild type (9)	1.3 ± 0.4	817±231	0.58 ± 0.14	21.4 ± 0.7

* Significantly increased versus wild type: $p < 0.001$.

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Table 3

Turnover of Hcy-thiolactone and total Hcy in mouse blood *in vivo*. *L*-Hcy-thiolactone was injected i.p. (600nmol/g body weight). Half-lives ($t_{0.5}$ = 0.69/k) and plasma concentrations at time zero (HTL ^{0}, tHcy^0) were calculated from the plasma concentrations at time t (HTL^t, tHcy^t) according to the equation $[HTL^t] = [HTL^0] \cdot e^{-k \cdot t}$ or $[tHcy^t] = [tHcy^0] \cdot e^{-k \cdot t}$, where k is a first order rate constant

Genotype	Diet	HTL^0, μM	HTL $t_{0.5}$, min (n)	tHcy^0, μM	tHcy $t_{0.5}$, min (*n*)
Pon1 $^{-/-}$	Control	34.1 ± 9.5	5.9 ± 1.2(6)	256 ± 23	27.1 ± 6.3 (4)
Wild type	Control	39.0 ± 13.9	5.0 ± 0.9 (15)	524±136	26.2 ± 2.6 (5)

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Table 4

Pon1 protects against Hcy-thiolactone neurotoxicity in mice. *L*-Hcy-thiolactone was injected i.p. (3,700 nmol/g body weight) and the mice were monitored for 90 min

Genotype (*n*)	Incidence of seizures, % (*n*)	Incidence of death, %	Seizure latency period, min	Death latency period, min
Pon1$^{-/-}$ (36)	52.8 (19) *	8.3 (3)	31.8±11.6‡	50±30
Wild type (44)	29.5 (13)	2.3 (1)	41.2±10.8	61

* Significantly different from wild type – Fisher exact test $p = 0.042$ versus wild type

‡ *T*-test $p = 0.019$ versus wild type.