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Functional promoter polymorphisms direct the expression of cystathionine gamma-lyase gene in mouse models of essential hypertension

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ABSTRACT

Despite the well-known role of cystathionine γ-lyase (Cth) in cardiovascular pathophysiology, transcriptional regulation of *Cth* remains incompletely understood. Sequencing of the *Cth* promoter region in mouse models of genetic/essential hypertension (viz. Blood Pressure High [BPH], Blood Pressure Low [BPL] and Blood Pressure Normal [BPN] mice) identified several genetic variations. Transient transfections of BPH/BPL-*Cth* promoter-reporter plasmids into various cell types revealed higher promoter activity of BPL-*Cth* than that of BPH-*Cth*. Corroboratively, endogenous *Cth* mRNA levels in kidney and liver tissues were also elevated in BPL mice. Computational analysis of the polymorphic *Cth* promoter region predicted differential binding affinity of c-Rel, HOXA3 and IRF1 with BPL/BPH-*Cth* promoter domains. Over-expression of c-Rel/HOXA3/IRF1 modulated BPL/BPH-*Cth* promoter activities in a consistent manner. Gel shift assays using BPH/BPL-*Cth*-promoter oligonucleotides with/without binding sites for c-Rel/HOXA3/IRF1 displayed formation of specific complexes with c-Rel/HOXA3/IRF1; addition of antibodies to reaction mixtures resulted in supershifts/inhibition of Cth promoter-transcription factor complexes. Furthermore, chromatin immunoprecipitation (ChIP) assays proved differential binding of c-Rel, HOXA3 and IRF1 with the polymorphic promoter region of BPL/BPH-*Cth*. Tumor necrosis factor-α (TNF-α) reduced the activities of BPL/BPH-*Cth* promoters to different extents that were further declined by ectopic expression of IRF1; on the other hand, siRNA-mediated down-regulation of IRF1 rescued the TNF-α-mediated suppression of the BPL/BPH-*Cth* promoter activities. In corroboration, ChIP analysis revealed enhanced binding of IRF1 with BPH/BPL-*Cth* promoter following TNF-α treatment. BPL/BPH-*Cth* promoter activity was diminished upon exposure of hepatocytes and cardiomyoblasts to ischemia-like pathological condition due to reduced binding of c-Rel with BPL/BPH-*Cth*-promoter. Taken together, this study reveals the molecular basis for the differential expression of *Cth* in mouse models of essential hypertension under basal and pathophysiological conditions.

1. Introduction

Essential hypertension (EH) is the most common risk factor for cardiovascular diseases with a prevalence of nearly 30% worldwide, and it aggregates with other cardiovascular risk factors. Even though the exact molecular mechanisms for its pathogenesis remain unclear, genetic studies have identified several candidate genes for EH. Cystathionine γ-lyase (*Cth*) is a new candidate gene for EH and it encodes a pyridoxal 5′ phosphate (PLP)-dependent enzyme involved in the trans-sulfuration pathway of amino acid metabolism [1]. Cth is an endogenous producer of H_2S [2–4], which is considered as the third gasotransmitter with a major role in cardiovascular system [1,3,5] in view of its ability to relax blood vessels via activation of ATP-dependent potassium channels (K_{ATP} channels) and membrane hyperpolarization of vascular smooth muscle cells [5,6]. *Cth* is expressed in various

Abbreviations: β-gal, beta-galactosidase; BPH, Blood Pressure High; BPL, Blood Pressure Low; BPN, Blood Pressure Normal; ChIP, chromatin immunoprecipitation; Cth, cystathionine γ-lyase; EH, essential hypertension; EMSA, electrophoretic mobility shift assay; HEK-293, human embryonic kidney-293; HOXA3, homeobox A3; IRF1, interferon regulatory factor 1; LOD, logarithm of odds; NF-κb, nuclear factor-kappa B; QTL, quantitative trait loci; RGD, Rat Genome Database; SHR, spontaneously hypertensive rats; SNP, single nucleotide polymorphisms; Sp1, specificity protein 1; TNF-α, tumor necrosis factor-alpha; TNFR, tumor necrosis factor receptor; VISTA, visualization tools for sequence alignments; WKY, Wistar-Kyoto rats.

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tissues/cell types including vascular smooth muscle/endothelial cells, liver, kidney, pancreas, prostate, heart and brain [7–12]. Genetic deficiency of *Cth* in mice exhibited age-dependent hypertension; the endogenous H_2S levels in aorta and heart of homozygous and heterozygous *Cth* knockout mice were reduced by about 80% and 50%, respectively; similarly, the serum H_2S levels in homozygous and heterozygous *Cth* knockouts were also diminished by 50% and 20%, respectively [1]. Over-expression of *Cth* protects against renal/myocardial ischemic-reperfusion injury likely by modulating the oxidative stress to preserve the mitochondrial structure and function [13,14]. Hence, *Cth* has emerged as a potential regulator in the pathogenesis of cardiovascular disorders. Multiple mutations in *Cth* revealed genetic basis of cystathioninuria [15] while a common non-synonymous single nucleotide polymorphism (SNP) 1364 T/T homozygote's in exon 12 was significantly associated with higher mean plasma total homocysteine [16]. The role of human *Cth* polymorphisms in relation to hypertension has not been studied till date apart from a population study in a Northern Chinese Han population in which the SNPs rs482843 and rs1021737 of the *Cth* gene were not associated with EH [17].

In various animal models of hypertension, endogenous *Cth* expression as well as H_2S production were diminished and exogenous administration of H_2S donor prevented the elevation of blood pressure [5,18–20]. Therefore, it might be possible that genetic variations in the *Cth* may influence the blood pressure homeostasis in mouse models of essential hypertension. In the current study, we sequenced the mouse *Cth* promoter in the genetically hypertensive BPH (Blood Pressure High), genetically normotensive BPN (Blood Pressure Normal) and genetically hypotensive BPL (Blood Pressure Low) mice strains and discovered a number of variations (A-476C, A-477C, A-486ΔA, C-487ΔC and A-488C) in BPL. Our results reveal that two promoter variations (A-476C, A-477C) alter the binding affinities of several transcription factors (viz. homeobox A3 [HOXA3], c-Rel and interferon regulatory factor 1 [IRF1]) and modulate *Cth* expression in these mouse models of EH. In addition, this study, for the first time, provides evidence and mechanism of regulation of *Cth* expression during inflammation and ischemia-like pathophysiological conditions.

2. Results

2.1. Comparative genomics analysis of mouse and rat Cth gene sequences

Although mouse models are highly useful to dissect the genetic basis of complex human diseases like essential hypertension and various resources are available for genetic mapping in mice [21], most of the quantitative trait loci (QTL) analyses have been performed in rats because of technical difficulties in measuring the cardiovascular phenotypes in mice. We probed the Rat Genome Database (RGD) [22] for blood pressure QTLs on *Cth*-harboring chromosome 2 and detected several blood pressure QTLs (Fig. 1A). The blood pressure-QTLs and respective LOD [logarithm (base 10) of odds] scores retrieved from RGD are shown in Table S1. Interestingly, the QTL Bp175 (248,961,074–283,538,297 bp) that harbors *Cth* gene (282,366,125–282,392,112 bp, RGD ID: 2443) displayed a significant linkage (LOD score = 3.46) with mean arterial blood pressure (Fig. 1A). Moreover, consistent with synteny/concordance between rat and mouse QTLs, [23–25] alignment of the mouse and rat genomic regions at *Cth* locus using mVISTA demonstrated >75% homology between these rodents at exons, introns and untranslated regions (Fig. 1B). While the extent of homology between each of the twelve *Cth* exons in mouse and rat were, in general, higher (>85%) than the noncoding regions, the exons 4, 6, 10 and 11 showed ~90% homology (Fig. 1B). Thus, *Cth* appeared as a logical candidate gene for further studies in mouse models of hypertension.

Table 1
Wild-type and mutant oligonucleotides used for EMSA experiments.

Name of the oligonucleotide	Sequence (5′ to 3′)
BPL-*Cth*-Wt-FP	AAAACAAAAA**AAACCCAAA**GT**T**TTAGGTTCGAC
BPL-*Cth*-Wt-RP	GTCGAACCTAAAACTTTGGGTTTTTTTTGTTTT
BPL-*Cth*-Mut-FP	AAAACAAAAA**CCCGAGTGG**GT**A**TTAGGTTCGAC
BPL-*Cth*-Mut-RP	GTCGAACCTAATACCCACTCGGGTTTTTGTTTT
BPH-*Cth*-Wt-FP	AAACAAAAAAA**AAAA**C**A**AA**GTTT**TAGGTTCGAC
BPH-*Cth*-Wt-RP	GTCGAACCTAAAACTTTGTTTTTTTTTTGTTT
BPH-*Cth*-Mut-FP	AAACAAAAAAA**TACG**C**C**AATAGGTTCGAC
BPH-*Cth*-Mut-RP	GTCGAACCTATTGGCGTATTTTTTTGTTT

The oligonucleotides are named as. Wt: wild-type, Mut: mutant, FP: forward primer, RP: reverse and complementary primer. The mutated nucleotides are indicated in bold and underlined. The bold nucleotides (without underline) in BPH-*Cth*-Wt-FP were deleted to design BPH-*Cth*-Mut-FP.

2.2. Endogenous expression of Cth in BPH and BPL kidney and liver

To study whether the endogenous *Cth* mRNA and protein expression differs between genetically hypertensive BPH and genetically hypotensive BPL mice, we investigated *Cth* mRNA and protein levels in liver and kidney tissues because kidney and liver are the main organs that express *Cth* in abundance besides vasculature [10]. Our qPCR analysis revealed that the *Cth* mRNA expression was significantly diminished in BPH (as compared to BPL mice) in both the tissue types: liver (~1.8-fold, $p < 0.05$) and kidney (~2.5-fold, $p < 0.01$) (Fig. 2A and B). Cth protein levels in BPH was also diminished (~2-fold) as compared to BPL in liver (Fig. 2C); however, Cth protein levels in BPH and BPL kidney tissues were similar (Fig. 2D).

2.3. Discovery of variations in BPL-Cth promoter and conservation of nucleotide sequences at the polymorphic domain among different mammals

To investigate the molecular mechanism of differential expression of *Cth* between BPH and BPL mice, ~1 kb promoter regions in these mouse models of hypertension were sequenced. Alignment of BPH-, BPL-, and BPN-*Cth* promoter sequences using ClustalW [26] led to the identification of several nucleotide variations: A-476C, A-477C, A-486ΔA, C-487ΔC and A-488C in BPL-*Cth* promoter with respect to BPH/BPN-*Cth* promoters (Fig. 3A).

To analyze if there is any conservation at the polymorphic domain of mouse *Cth* promoters, alignment of relevant BPL/BPH-*Cth* promoter sequence and *Cth* promoter sequences of various mammalian species (human, rat, chimpanzee, cow and orangutan) were carried out using the GeneDoc software [27] (Fig. 3B). Interestingly, the C-allele discovered at the −476 bp position in BPL-*Cth* promoter was present at the corresponding positions in *Cth* promoter of human, chimpanzee and cow; the C-allele at the −477 bp position in BPL-*Cth* was observed only in cow *Cth* promoter among the mammals included in this analysis (Fig. 3B).

2.4. Differential activities of the Cth promoters under basal conditions

To assess the functional implications of variations present in the BPL-*Cth* promoter, the BPH-*Cth* and BPL-*Cth* reporter constructs as well as pGL3-Basic plasmid (as negative control) were transfected into BRL-3A, HEK-293, N2a and H9c2 cells (Fig. 4). In all the tested cell lines, BPL-*Cth* promoter activity was significantly higher than that of BPH-*Cth*: ~1.4-fold in BRL-3A (one-way ANOVA $F = 262.0$, $p < 0.0001$, Fig. 4B), ~1.5-fold in HEK-293 (one-way ANOVA $F = 180.2$, $p < 0.0001$, Fig. 4C), ~2.3-fold in in N2a (one-way ANOVA $F = 155.3$, $p < 0.0001$, Fig. 4D), and ~1.9-fold in H9c2 (one-way ANOVA $F = 280.9$, $p < 0.0001$, Fig. 4E). Interestingly, the differential *Cth* promoter