Supporting Information

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SI Materials and Methods

Targeted Mutagenesis of the Mouse Carbonic Anhydrase 5A Gene. A phage genomic library from the 129/Sv mouse strain (Stratagene and Agilent Technologies, Inc.) was screened using the murine carbonic anhydrase 5A (*Car5A*) cDNA as a probe. Five overlapping mouse genomic fragments covering the entire *Car5A* gene of ~25 kb were isolated and subcloned into pBluescript KS vector (Promega). To disrupt the *Car5A* gene, we used a plasmid (a gift from Richard Proia, National Institutes of Health, Bethesda, MD) containing both the neomycin-resistance (Neo) gene and the Herpes simplex virus thymidine kinase (TK) gene (1). A 4.5-kb SalI-XhoI fragment containing part of intron 2 was inserted into the XhoI site between the TK and Neo genes of PGKNeobpATK. A 3.5-kb HindIII-XbaI fragment containing approximately half of intron 3 and exons 4–6 was subcloned into the SalI site downstream of the Neo gene. The targeting vector that we generated replaced a 3.2-kb fragment encompassing 150 bp of intron 2, all of exon 3, and 3.05 kb of intron 3 with a 1.7-kb PGKneo cassette. The construct deletes exon 3 containing two of the three Zn-binding histidine residues and introduces a frameshift beginning in exon 4 that should create a null mutation. The targeting vector (25 μg) was linearized with NotI and introduced into the 129/Sv-derived ES cell line RW4 (Genome Systems, Inc.) (1×10^7 cells) by electroporation (230 V and 500 μF) in a Bio-Rad gene Pulser. After 24 h, the cells were placed under selection with 400 μg/mL G418 (GIBCO-Life Technologies) and 2 μM ganciclovir (Syntex) for 6 d. Genomic DNA of resistant clones was digested with BglII and hybridized with a 0.9-kb XbaI-XhoI external probe.

Two independent, targeted ES clones were injected into the blastocysts of C57BL/6J mice and transferred into pseudopregnant female mice as described (2). Chimeric male offspring were bred to C57BL/6J females and the agouti F_1 offspring were tested for transmission of the disrupted allele by Southern blot analysis of BglII-digested genomic DNA. Heterozygous matings of the F_1 mice were carried out to produce homozygous F_2 mutant mice.

The *Car5A* knockout (KO) offspring were genotyped by PCR analysis of tail genomic DNA. The PCR conditions included 33 cycles at 94 °C for 30 s, 65 °C for 40 s, and 68 °C for 3 min with a 5-s autoextension, using 5′-ACTGCACACTTCCTCCAACAAGCCCACC and 5′-TGACCGCTTCCTCGTGCTTTACGGTATCGCCGC as forward primers for WT and mutant alleles, respectively, and 5′-ATGACCGCCAGCCCGTTCTCC as reverse primer for both. Products obtained from the KO and WT alleles were 550 and 603 bp, respectively.

Targeted Mutagenesis of the Mouse Carbonic Anhydrase 5B Gene. With a 1-kb *Car5B* cDNA supplied by us, Genome Systems screened a BAC library and provided a 35.3-kb genomic clone of mouse carbonic anhydrase 5B (*Car5B*). A shotgun approach was used to determine the organization of *Car5B* gene. The fragments generated by restriction digestion were subcloned into pZero vector. Using 18- and 6-kb KpnI fragments and a 12-kb SacI fragment as well as the original clone as templates, the entire gene was PCR-amplified with exonic primers. The exon/intron boundaries were determined by sequencing. More recently, the information from the mouse genomic database revealed that the mouse *Car5B* gene is 48.9 kb and has an additional 5′-exon designated as noncoding exon 1.

To generate *Car5B* KO mice, we constructed a replacement targeting vector by ligating a 3.1-kb KpnI-XbaI fragment containing ~3 kb of intron 2 and 36 bp of coding exon 3 of the *Car5B* gene on one side of the *Neo* gene of the targeting vector pPNT-Cass *Lox*A (2, 3) and a 4.5-kb XhoI-NotI genomic fragment including 38 bp of bacterial sequence from vector pZero and extending from 67 bp of coding exon 3 to coding exon 6 on the other side of the *Neo* gene. This vector contains a cassette flanked by *Lox*P recombination site elements, including sequences conferring neomycin resistance and expressing cre recombinase under direction of a testis-specific angiotensin-converting enzyme promoter (3). After expression of cre recombinase in the germ line of the male chimeric transgenic mice, the cassette was excised from the genome leaving one *Lox*P site behind. In this construct, we replaced 10 amino acids of coding exon 3 of the *Car5B* allele with the tACE-CRE-neo^r insert and the 38 bp of pZero. The deletion included two of the three Zn-binding histidine residues (H94 and H96) and introduced a frameshift beginning in coding exon 4 and a stop codon after 11 missense amino acids, including the third Zn^{2+}-binding histidine, H119, in coding exon 4. The introduction of a linearized targeting vector into the 129S1/Sv-$^+$p–derived ES cell line W9.5s (Yale University, the Jackson Laboratory, catalog no. 000090, 1×10^7 cells) and positive/negative selection were as described earlier. Southern blot analysis was performed on genomic DNA from resistant clones. The genomic DNA was digested with SacI and KpnI restriction enzymes and probed with an external probe of 1 kb. Cells from two independent homologous recombinant clones were microinjected into blastocysts and transferred into pseudopregnant mice. The resulting chimeric offspring were bred to C57BL/6J mice. The agouti F_1 offspring were tested for the deletion as well as for the removal of the selection cassette by PCR and direct sequencing of the PCR product. F_1 matings produced hemizygous males and homozygous female F_2 mutant mice (*Car5B* gene being X-linked).

PCR conditions for genotyping of *Car5B* KO mice included 10 cycles at 95 °C for 15 s, 65 °C for 40 s, and 68 °C for 1 min and 20 cycles of 95 °C for 15 s, 65 °C for 40 s, and 68 °C for 1 min with a 5-s autoextension with 5′-TGCTGGATGTTTTCTTTCAGTGGTCGAGG (forward primer) and 5′-ACCAATCACTCTTCACCCACCATTCTCAAGCC (reverse primer). Products obtained from the KO and WT alleles were 522 and 436 bp, respectively. Their identities were confirmed by direct sequencing.

1. Yamanaka S, et al. (1994) Targeted disruption of the Hexa gene results in mice with biochemical and pathologic features of Tay-Sachs disease. *Proc Natl Acad Sci USA* 91(21):9975–9979.
2. Fleming RE, et al. (2002) Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. *Proc Natl Acad Sci USA* 99(16):10653–10658.
3. Bunting M, Bernstein KE, Greer JM, Capecchi MR, Thomas KR (1999) Targeting genes for self-excision in the germ line. *Genes Dev* 13(12):1524–1528.

Table S1. Comparison of urinary metabolites in wild-type (WT), CA VB, and CA VA mice (mmol/mol creatinine)

Metabolite	WT (9)	CA VB (8)	CA VA (4)
Lactate	103.74 ± 81	170.8 ± 121 ($P < 0.20$)	186 ± 20 ($P < 0.12$)
Fumarate	226.50 ± 147	220.0 ± 124 ($P < 0.92$)	**2763 ± 557 ($P < 0.0001$)**
Malate	255 ± 324	170 ± 56 ($P < 0.08$)	**3246 ± 88 ($P < 0.0001$)**
3-Hydroxybutyric	11.2 ± 11	13.7 ± 11 ($P < 0.65$)	11.8 ± 1.0 ($P < 0.92$)
Acetoacetate	1.5 ± 1.7	**11.42 ± 12.7 ($P < 0.04$)**	**28.2 ± 3.6 ($P < 0.0001$)**
α-Ketoglutarate	332 ± 339	634.6 ± 451 ($P < 0.14$)	**8497 ± 598 ($P < 0.0001$)**
Adipic	25 ± 20	36.9 ± 20.7 ($P < 0.25$)	47.7 ± 6.0 ($P < 0.06$)
Suberic	5.7 ± 3	17.7 ± 18.9 ($P < 0.08$)	**20.2 ± 2 ($P < 0.0001$)**
Sebacic	0.5 ± 1.3	25.1 ± 39.3 ($P < 0.08$)	**11.4 ± 2.6 ($P < 0.0001$)**

Boldface values are significant. Adult mice of the genotypes indicated were fed on chow diets and received water without citrate supplement for at least 2 wk before urine collections. Urine was collected from each mouse studied between 1600 and 1700 hours onto a Parafilm, transferred by pipette to a microcentrifuge tube, and stored frozen until the pooled sample from the 3-d collection was delivered to the metabolic screening laboratory. Each sample was treated with urease and analyzed by the method of Shoemaker for carbohydrates, organic acids, and amino acids following trimethylsilylation by tandem mass spectrometry (1, 2). *P* values indicate significance levels determined by paired *t*-test comparisons with wild-type. Values in parentheses indicate *n* numbers for each mouse type.