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Limited Accumulation of Damaged Proteins in L-Isoaspartyl (D-Aspartyl) *O*-Methyltransferase-deficient Mice*

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L-Isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT1) can initiate the conversion of damaged aspartyl and asparaginyl residues to normal L-aspartyl residues. Mice lacking this enzyme (*Pcmt1*−/− mice) have elevated levels of damaged residues and die at a mean age of 42 days from massive tonic-clonic seizures. To extend the lives of the knockout mice so that the long term effects of damaged residue accumulation could be investigated, we produced transgenic mice with a mouse *Pcmt1* cDNA under the control of a neuron-specific promoter. *Pcmt1* transgenic mice that were homozygous for the endogenous *Pcmt1* knockout mutation ("transgenic *Pcmt1*−/− mice") had brain PCMT1 activity levels that were 6.5–13% those of wild-type mice but had little or no activity in other tissues. The transgenic *Pcmt1*−/− mice lived, on average, 5-fold longer than nontransgenic *Pcmt1*−/− mice and accumulated only half as many damaged aspartyl residues in their brain proteins. The concentration of damaged residues in heart, testis, and brain proteins in transgenic *Pcmt1*−/− mice initially increased with age but unexpectedly reached a plateau by 100 days of age. Urine from *Pcmt1*−/− mice contained increased amounts of peptides with damaged aspartyl residues, apparently enough to account for proteins that were not repaired intracellularly. In the absence of PCMT1, proteolysis may limit the intracellular accumulation of damaged proteins but less efficiently than in wild-type mice having PCMT1-mediated repair.

The spontaneous chemical modification of proteins by reaction with oxygen, water, sugars, and other abundant metabolites is unavoidable. The accumulation of such nonenzymatically altered proteins is associated with normal aging as well as atherosclerosis, Alzheimer's disease, and diabetes (1–3). Organisms have several strategies for dealing with damaged proteins, including intracellular proteolysis mediated by proteasome and lysosome action (4–6). Some types of covalent damage, however, are simple enough to recognize and repair directly (7). Enzymes such as prolyl *cis-trans* isomerase (8), methionine sulfoxide reductase (9), and disulfide isomerase (8) can restore activity to proteins that have been chemically altered.

We are interested in a common type of spontaneous protein damage in which L-aspartyl and L-asparaginyl residues undergo an intramolecular reaction that converts them to L-succinimidyl residues (10, 11). Nonenzymatic hydrolysis of the succinimide ring readily occurs at either carbonyl to generate both normal aspartyl residues and isoaspartyl residues, in which the peptide backbone proceeds through the β-carbonyl rather than the α-carbonyl moiety (12). The succinimide also racemizes more rapidly than do the open chain forms, and hydrolysis of the D-succinimide produces D-aspartyl and D-isoaspartyl residues (12). Local protein structure causes some L-aspartyl and L-asparaginyl residues to be especially prone to succinimide formation, and the presence of damaged aspartyl residues at these sites can significantly alter the structure, function, and immunogenicity of the protein (10, 13).

To minimize the accumulation of damaged aspartyl residues in cellular proteins, all mammalian tissues possess an L-isoaspartyl (D-aspartyl) *O*-methyltransferase (EC 2.1.1.77; designated PCMT1[1] in mice) (14). This enzyme uses *S*-adenosyl-L-methionine (AdoMet) to methylate L-isoaspartyl (and, less efficiently, D-aspartyl) residues but not normal L-aspartyl residues (15). Nonenzymatic deesterification of the methylated residues returns them to the succinimide form much more rapidly than occurs in the absence of methylation, resulting in the eventual conversion of most of the damaged residues to the "repaired" L-aspartyl form (16, 17).

The physiological importance of this pathway remained unclear until *Pcmt1* knockout (*Pcmt1*−/−) mice were created and found to display a distinctive phenotype (18, 19). *Pcmt1*−/− mice have 2–6-fold higher levels of damaged aspartyl residues in their brain, heart, liver, and erythrocytes than are observed in wild-type tissues (18). Furthermore, *Pcmt1*−/− mice are smaller than their *Pcmt1*+/− and *Pcmt1*+/+ littermates, undergo severe tonic-clonic seizures, and die at an average age of 42 days (18, 19). Electroencephalographic analysis shows that these mice suffer abnormal brain activity about 50% of the time, not just during the tonic-clonic seizures (20). Administration of the anti-seizure drug valproic acid enabled *Pcmt1*−/− mice to attain the same size and weight as their wild-type littermates, suggesting that the absence of the methyltransferase did not interfere directly with food intake or metabolism (20). The combination of valproic acid and clonazepam prolonged mean survival but only by 36 days (20).

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[1] The abbreviations used are: PCMT1, L-isoaspartyl (D-aspartyl) *O*-methyltransferase; AdoMet, *S*-adenosyl-L-methionine; [^{14}C]AdoMet, *S*-adenosyl[*methyl*-^{14}C]-L-methionine; NSE, neuron-specific enolase; BisTris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol.

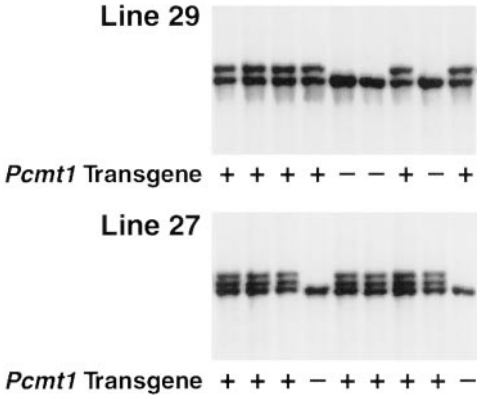

FIG. 1. **Identification of transgenic mice from two different *Pcmt1* transgenic mouse lines.** Mouse genomic DNA was digested with *Eco*RI and examined by Southern blotting with a neuron-specific enolase promoter probe. Nontransgenic mice had a single band corresponding to the endogenous enolase gene. Transgenic mice were identified by the presence of additional bands (two larger bands in the case of *line 27* and one larger band in the case of *line 29*).

These results have raised new questions. Does the repair methyltransferase, although expressed in all tissues, have particular importance within the brain? Is it important in neurons or in nonneuronal cell types? If the seizures in the *Pcmt1*−/− mice were prevented, would other organs function abnormally as damaged aspartyl residues accumulated? In the current study, we have approached these questions by producing transgenic mice that express Pcmt1 solely within neurons.

EXPERIMENTAL PROCEDURES

Generation of Pcmt1 Transgenic Mice—A rat neuron-specific enolase (NSE) promoter was used to direct the expression of mouse *Pcmt1* cDNA in the brains of transgenic mice. The methyltransferase coding sequence (including 119 base pairs of 5′-noncoding and 777 base pairs of 3′-noncoding sequence) was obtained from a 1580-base pair murine testis cDNA clone (21) and was removed from plasmid sequences with *Eco*RI. The proximal rat NSE promoter was isolated from the plasmid NSE-APP695 (22) after digestion with *Hin*dIII. After overhangs were filled with Klenow polymerase, the mouse *Pcmt1* cDNA and the rat NSE vector were ligated, and the NSE-*Pcmt1* transgenic construct was isolated by digestion with *Sal*I. The transgene (2 ng/μl) was microinjected into F2 C57BL/6 × SJL fertilized mouse eggs by standard techniques (23). From 37 microinjected eggs, 33 pups were obtained, and 4 harbored the *Pcmt1* transgene. These transgenic founders were identified by polymerase chain reaction with primers corresponding to mouse *Pcmt1* cDNA sequences (5′-GCCAGCCACTCGGAGCTAATCC-3′ from exon 1 and 5′-CCACTATTTCCAACCATCCGTGC-3′ from exons 4 and 5). Southern blot analysis of tail DNA confirmed the integration of the transgene (Fig. 1). Two of these mice, founders 27 and 29, were bred with C57BL/6 × 129/SvJae mice that were heterozygous for a knockout mutation in the endogenous *Pcmt1* gene (18). DNA samples from the tails of these mice were genotyped by polymerase chain reaction, both to detect the transgene and to detect the knockout mutation. Mice that were heterozygous for the knockout mutation and hemizygous for the transgene were selected for breeding. All mice were weaned at 21 days of age, housed in a barrier facility with a 12-h light/dark cycle, and fed a chow diet.

Preparation of Mouse Tissues—Brain, heart, liver, kidney, and testis were removed immediately from sacrificed animals and placed in ice-cold buffer (5 ml/g wet weight) containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4, and the protease inhibitor phenylmethylsulfonyl fluoride (25 μM). The tissues were disrupted in a glass homogenization tube with a Teflon pestle rotating at 310 rpm for four 10-s intervals. The homogenates were placed in 1.5-ml tubes and centrifuged at 20,800 × *g* for 10 min. The resulting supernatant fractions contained both cytosolic proteins and microsomes and were kept frozen until used.

Whole blood (100–200 μl) was taken from the tail or heart and combined with 200 μl of 2 mg/ml disodium EDTA, 10 mM sodium phosphate, 137.9 mM sodium chloride, pH 7.4. Erythrocytes were collected by centrifugation at 4000 × *g* for 4 min and washed four times with 1 ml of the above buffer. Pelleted erythrocytes were lysed in 5 volumes of 5 mM sodium phosphate, 1 mM disodium EDTA, pH 7.4, and 25 μM phenylmethylsulfonyl fluoride. The lysates were centrifuged in 1.5-ml conical tubes at 20, 800 × *g* for 10 min to remove the membrane ghosts, and the supernatant fractions were stored at −20 °C.

Protein Assay—A modified Lowry assay was used to determine protein concentrations in the extracts. Assays were done in duplicate with bovine serum albumin as a standard (18).

Assay of L-Isoaspartyl (D-Aspartyl) O-Methyltransferase Activity—Methyltransferase activity was assayed by its ability to transfer the methyl group from *S*-adenosyl-L-methionine to ovalbumin. The supernatant fraction from homogenized tissues (10–60 μg of brain, heart, or testis protein; 0.6–0.8 mg of erythrocyte protein) was incubated with 0.8 mg of ovalbumin (Sigma, Grade V) in 0.2 M BisTris-HCl, pH 6.0, containing 10 μM [^{14}C]AdoMet (53 mCi/mmol; Amersham Pharmacia Biotech) in a final volume of 40 μl at 37 °C for 15 min. NaOH (80 μl of a 200 mM solution) was added to stop the reaction and to hydrolyze the [^{14}C]methyl esters formed on ovalbumin to [^{14}C]methanol. The reaction mixture was immediately spotted onto an 8 × 2-cm piece of thick filter paper and incubated *above* 5 ml of Safety-Solve scintillation fluid (Research Products International) in the neck of a sealed 20-ml scintillation vial at room temperature for 2 h to allow [^{14}C]methanol to diffuse into the scintillation fluid. The filter paper was removed, and the scintillation fluid was counted for radioactivity. Incubations containing *S*-adenosyl-L-[*methyl*-^{14}C]methionine, ovalbumin, and buffer constituted the "blank" for the assay; the radioactivity in these tubes (typically <5% of the nonblank samples) was subtracted from total counts in the determination of enzyme activity.

Quantitation of Damaged Aspartyl Residues—Cellular proteins were incubated at 37 °C for 2 h with 0.8 μg of recombinant human L-isoaspartyl methyltransferase (specific activity, 10,000 pmol of methyl esters formed on ovalbumin at 37 °C/min/mg protein) (24) in 0.2 M BisTris-HCl, pH 6.0, and 10 μM [^{14}C]AdoMet in a final volume of 40 μl. After base hydrolysis, [^{14}C]methanol production was measured as described above to quantitate L-isoaspartyl and D-aspartyl methyl-accepting sites in cellular proteins. Incubations containing [^{14}C]AdoMet, recombinant methyltransferase, and buffer constituted the blank for the assay; the radioactivity in these tubes was subtracted from each sample's total counts. Each sample was assayed in duplicate or triplicate, and the average value is reported.

Quantitation of Endogenously Methylated Damaged Aspartyl Residues—Damaged residues that are already methylated within cells by the endogenous methyltransferase and *S*-adenosyl-L-methionine are not measured in the assay described above but can be quantified after mild base treatment. Protein (8.3–9.6 mg) from homogenized *Pcmt1*−/− and *Pcmt1*+/+ brains was incubated in 20 μl of 75 mM potassium borate, pH 10.2. After times ranging from 5 s to 360 min, 10 μl of 500 mM BisTris-HCl, pH 5.7, was added to lower the pH to about 6. Then, recombinant human methyltransferase (5 pmol/min) and *S*-adenosyl-L-[*methyl*-^{14}C]methionine (10 μM final concentration) in 10 μl of 150 mM BisTris-HCl, pH 6.0, was added, and these reaction mixtures were incubated at 37 °C for 135 min. The reaction was stopped by freezing on dry ice, and then the base-labile methyl esters were quantitated as described above.

Urine Collection and Analysis—Urine, freshly voided on Parafilm, was collected with a pipette and stored frozen until used. Creatinine in the urine was measured by a modified form of the procedure of Bosnes and Taussky (25). An aliquot of each urine sample (0.3–1 μl) or standard creatinine (0–25 μg) was diluted to 50 μl with water in duplicate tubes. Picric acid was added (25 μl of a 40 mM solution), and the tubes were capped and incubated in a boiling water bath for 45 min. After cooling to room temperature, 25 μl of 0.75 M NaOH was added. Within 15 min, 90 μl of each sample was transferred to a flat-welled microtiter plate (Costar), and absorbance was measured at 525 nm with a Beckman DU-600 plate reader. Damaged aspartyl residues in the urine were assayed with recombinant human methyltransferase as described above.

Amino Acid Analysis—Free amino acids and isoaspartyl-containing dipeptides in urine were derivatized with *o*-phthalaldehyde and 2-mercaptoethanol, separated by reverse-phase high pressure liquid chromatography, and quantitated by fluorescence as described previously (26). A precipitate that formed upon mixing of the urine and derivatization reagent was removed by centrifugation at 20,800 × *g* for 3 min prior to injection. Urine that had been dried in 6 × 50-mm glass tubes was hydrolyzed in vaporized hydrochloric acid *in vacuo* at 108 °C for 3 h with a PicoTag Work Station (Waters); amino acids in the hydrolysates were quantitated as described above. The fluorescence color constants for these derivatives were determined with amino acid and dipeptide standards.

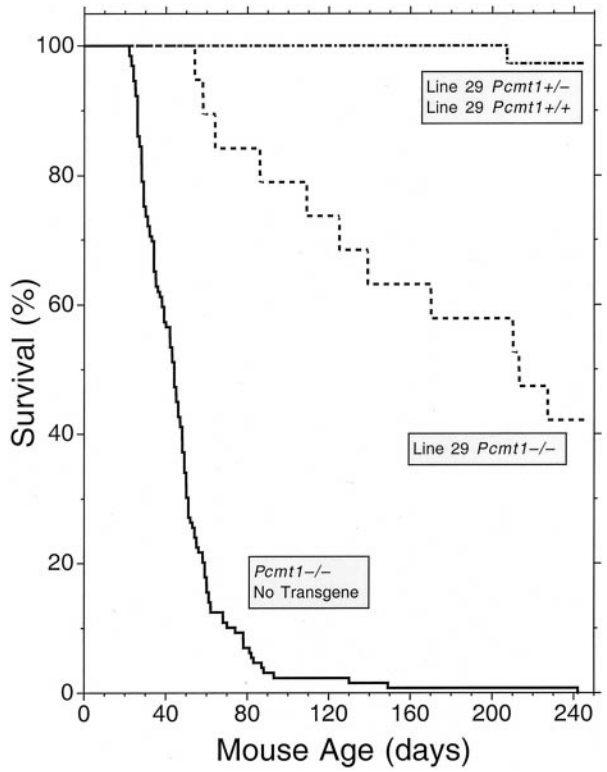

FIG. 2. **Enhanced survival of *Pcmt1*−/− mice expressing a *Pcmt1* transgene driven by a neuron-specific promoter.** Shown are data for *Pcmt1*−/− mice ($n = 129$) lacking the transgene (*solid line*) and for line 29 mice in the *Pcmt1*−/− background ($n = 19$; *dashed line*) or in the *Pcmt1*+/− or *Pcmt1*+/+ backgrounds ($n = 36$; *dashed-dotted line*). These data represent mice that died spontaneously or are still alive.

RESULTS

Expression of a Pcmt1 Transgene in Neurons Prolongs the Lives of Mice Lacking Endogenous Pcmt1—We generated two *Pcmt1* transgenic mouse lines, lines 27 and 29, in which the murine *Pcmt1* methyltransferase cDNA was placed under the control of a neuron-specific promoter. We then compared the survival of "transgenic *Pcmt1*−/− mice" with that of "nontransgenic *Pcmt1*−/− mice." Whereas nontransgenic *Pcmt1*−/− ($n = 129$) mice died at a median age of 44 days (with only one mouse living beyond 150 days), the transgenic *Pcmt1*−/− mice lived much longer (Fig. 2). Of 11 line 27 transgenic *Pcmt1*−/− mice examined in this study, 6 died between 30 and 90 days of age, but 4 lived from 549 to 757 days. Line 29 transgenic *Pcmt1*−/− mice ($n = 19$) died at a median age of 213 days (Fig. 2), and 3 lived more than 400 days. The nontransgenic *Pcmt1*−/− mice began to die at about 21 days. In contrast, none of the line 29 transgenic *Pcmt1*−/− mice died at less than 52 days of age (Fig. 2).

Line 27 and most of the line 29 mice possessing one or two copies of the endogenous *Pcmt1* gene were indistinguishable from comparable nontransgenic mice in size, weight, and behavior, although 15 of 94 line 29 mice ran rapidly in circles. Line 27 and line 29 mice appeared to have normal physiological functions and had unremarkable tissue histology. The transgenic *Pcmt1*−/− mice, however, differed from the nontransgenic *Pcmt1*−/− animals in several ways. First, although nontransgenic *Pcmt1*−/− mice weighed significantly less than age- and sex-matched *Pcmt1*+/− and *Pcmt1*+/+ littermates (18), the weights of transgenic *Pcmt1*−/− mice were identical to their *Pcmt1*+/− and *Pcmt1*+/+ littermates (data not shown). Second, due to their low grade seizure activity (*e.g.* facial grooming and myoclonic jerks), the nontransgenic *Pcmt1*−/− mice could often be distinguished by observation from wild-type and heterozygous littermates. In contrast, these abnormalities were not observed in the transgenic *Pcmt1*−/− mice.[2] Finally, nontransgenic *Pcmt1*−/− mice of either sex never produced litters, even when housed with *Pcmt1*+/+ animals and given the anti-seizure drugs valproic acid and clonazepam, and only a single mating was observed (20). Two line 27 *Pcmt1*−/− animals, however, produced two small litters without the administration of any drug treatments, and 17 pairings involving male and/or female line 29 *Pcmt1*−/− mice have produced three litters (from two different *Pcmt1*−/− mothers).

TABLE I
L-Isoaspartyl (D-aspartyl) O-methyltransferase activity in mice possessing the Pcmt1 transgene with a neuron-specific enolase promoter

Methyltransferase specific activities were determined as described under "Experimental Procedures" in duplicate or triplicate in each tissue for the number of mice given in parentheses. The value of standard deviation here represents differences between individual mice; the variation of the assay within a single tissue extract was generally very low (<5%).

Tissue	Genotype of the endogenous methyltransferase gene		
	Pcmt1−/−	*Pcmt1*+/−	*Pcmt1*+/+
	pmol methyl groups transferred to ovalbumin/min/mg protein		
Line 27			
Brain	1.22 ± 0.51 (12)	8.25 (1)	17.4 (1)
Heart	0.010 ± 0.001 (4)	3.50 ± 2.50 (2)	11.4 (1)
Testis	0.011 ± 0.005 (4)	9.03 ± 5.77 (2)	20.4 (1)
Erythrocyte	0.001 ± 0.000 (4)	0.50 ± 0.04 (2)	1.37 (1)
Liver	0.016 ± 0.005 (4)	0.431 (1)	1.1 (1)
Kidney	0.023 ± 0.016 (4)	0.69 ± 0.43 (2)	2.13 (1)
Line 29			
Brain	2.53 ± 0.87 (16)	10.6 ± 0.7 (5)	19.2 ± 0.5 (3)
Heart	0.022 ± 0.013 (4)	5.5 ± 2.1 (3)	11.0 ± 5.0 (3)
Testis	0.012 ± 0.006 (4)	6.6 ± 0.6 (3)	20.0 ± 3.4 (3)
Erythrocyte	0.002 ± 0.001 (4)	0.60 ± 0.05 (3)	1.3 ± 0.1 (3)
Liver	0.023 ± 0.008 (4)	0.51 ± 0.07 (3)	1.1 ± 0.1 (3)
Kidney	0.031 ± 0.006 (4)	0.84 ± 0.11 (3)	1.7 ± 0.2 (2)

Localization of Methyltransferase Activity in Tissues of Line 27 and Line 29 Transgenic Mice—The *Pcmt1* transgene controlled by a neuron-specific promoter appeared to rescue, at least partially, the early death phenotype seen in mice lacking the endogenous methyltransferase. We next investigated where in the mouse, and at what level, the transgene was being expressed by assaying methyltransferase activity in various mouse tissues. As expected, transgenic mice possessing one or two copies of the endogenous *Pcmt1* gene expressed the methyltransferase in all tissues assayed, including brain, heart, testes, erythrocytes, liver, and kidney, at levels similar to those observed in nontransgenic mice (Table I) (18). In contrast, transgenic *Pcmt1*−/− mice expressed methyltransferase activity in the brain but not in the other tissues (Table I), and Western blot analysis detected PCMT1 protein only in brain homogenates (data not shown), suggesting that the neuron-specific enolase promoter was properly directing expression to neurons. However, the activity of this transgene-derived methyltransferase in the brain was relatively low; line 27 *Pcmt1*−/− brains had only 6.5% and line 29 *Pcmt1*−/− brains only 13% of the PCMT1 activity observed in wild-type brains. The amount of activity in brains from young (50 days) and older (370 days) transgenic *Pcmt1*−/− animals was not significantly different (data not shown). Since about half of the cells in the brain are not neurons (27), some reduction of methyltrans-

[2] Nonfatal running/jumping seizures have been observed in three transgenic *Pcmt1*+/− and *Pcmt1*+/+ mice but never in nontransgenic animals.

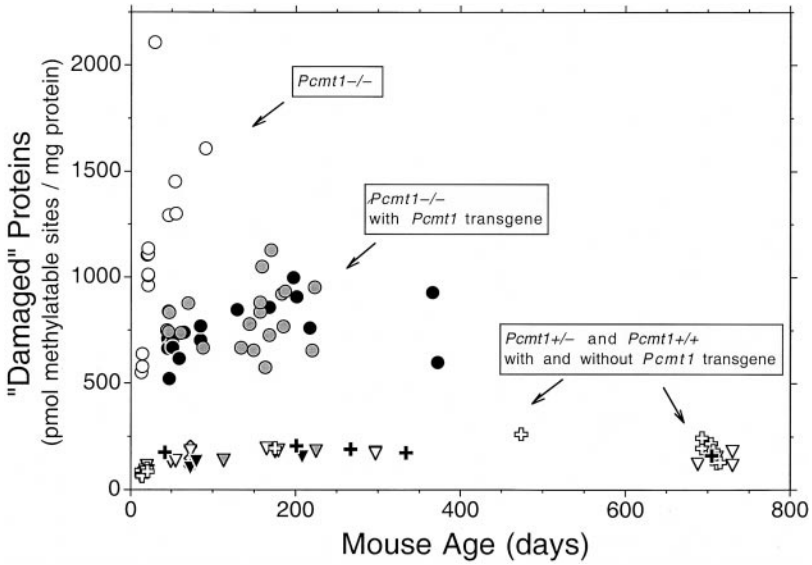

FIG. 3. **Accumulation of damaged aspartyl residues in brain cytosolic/microsomal polypeptides.** Recombinant human L-isoaspartyl (D-aspartyl) *O*-methyltransferase was used to quantitate damaged aspartyl residues in polypeptides that remain in the supernatant following a 20,800 × *g* centrifugation of whole brain homogenates as described under "Experimental Procedures." *Open circles, Pcmt1*−/− (n = 14); *gray circles,* transgenic line 27 *Pcmt1*−/− (n = 21); *black circles,* transgenic line 29 *Pcmt1*−/− (n = 18); *open crosses, Pcmt1*+/− (n = 14); *black crosses,* transgenic line 29 *Pcmt1*+/− (n = 5); *open triangles, Pcmt1*+/+ (n = 17); *open diamonds, Pcmt1*+/− or *Pcmt1*+/+ (n = 6); *gray triangles,* transgenic *line 27 Pcmt1*+/+ (n = 2); and *black triangles,* transgenic *line 29 Pcmt1*+/+ (n = 3).

ferase activity was expected in these brains. The low activity observed here, however, suggested that even in neurons the expression of the transgene was weaker than that of endogenous *Pcmt1* in wild-type neurons. Relatively low levels of neuronal expression from the neuron-specific enolase promoter have been reported by other investigators (28, 29).

Accumulation of Damaged Aspartyl Residues in Brain Tissue—The finding that transgenic mice expressing methyltransferase solely in neurons lived longer than nontransgenic knockout mice led us to compare the accumulation of damaged aspartyl residues in the brains of these animals. Recombinant human methyltransferase was used to label these residues in cytosolic/microsomal proteins with [^{14}C]methyl groups from [^{14}C]AdoMet *in vitro*. Examining 39 transgenic and 13 nontransgenic *Pcmt1*−/− mice, we found about 50% fewer damaged aspartyl residues when the transgene was present, indicating that the transgene-derived enzyme was repairing damaged neuronal proteins. However, the transgenic *Pcmt1*−/− brains still had about 4.5-fold more damaged residues than did *Pcmt1*+/− and *Pcmt1*+/+ brains (Fig. 3). These damaged residues could be accumulating both in neurons, due to the relatively low methyltransferase activity, and in glia, which should not express the transgene at all.

Examination of the levels of aspartyl damage in the brain with respect to age revealed several interesting points. First, these levels increased with age in young animals; 40-day-old and older mice had significantly more damaged residues per mg of protein than did the 13–21-day-old mice (p = 0.001 for *Pcmt1*−/− mice; $p = 10^{-8}$ for *Pcmt1*+/− and *Pcmt1*+/+ mice; Fig. 3). Second, the 13–14-day-old *Pcmt1*−/− animals already possessed about 8-fold more damaged aspartyl residues per mg protein than did age-matched *Pcmt1*+/− and *Pcmt1*+/+ animals (Fig. 3). The difference in the amount of damage remained 8–11-fold as these mice aged to 91 days, demonstrating that both nursing and weaned *Pcmt1*−/− mice accumulate these residues. Finally, there was no significant increase in the level of damaged aspartyl residues in transgenic *Pcmt1*−/− mice after about 100 days of age (Fig. 3). In addition, although line 29 *Pcmt1*−/− mice averaged twice as much brain PCMT1 activity as did comparable line 27 mice, the plateau level of damaged aspartyl residues in these two lines was not significantly different. The quantity of damaged residues in *Pcmt1*+/− and *Pcmt1*+/+ mice also attained an apparent steady state, although at a much lower level (Fig. 3). Because damaged residues were arising continuously in the cellular proteins, this stable level of damage probably represents a steady state between new damage, methyltransferase-linked repair, protein turnover, and perhaps unknown factors.

In control experiments, we asked whether the low level of damaged residues measured in the assays of *Pcmt1*+/− and *Pcmt1*+/+ mice resulted in part from the fact that some were already methylated by the endogenous enzyme. We therefore incubated brain cytosolic proteins under basic conditions where L-isoaspartyl α-methyl esters should hydrolyze within a few minutes to generate L-isoaspartyl residues with about an 80% yield (30, 31). Analysis of these samples with recombinant methyltransferase (data not shown) indicated that the true number of L-isoaspartyl residues in the *Pcmt1*+/− and *Pcmt1*+/+ proteins could be as much as 2.4-fold higher than the data shown in Fig. 3.[3] Coupled with the 8–11-fold higher levels actually observed, this result indicates that the level of damaged aspartyl residues in *Pcmt1*−/− brain proteins was at least 4-fold higher than in heterozygous and wild-type brain proteins.

Limited Accumulation of Damaged Aspartyl Residues in Heart, Testes, and Erythrocytes—The longer life span of the transgenic *Pcmt1*−/− mice enabled us to investigate whether accumulation of damaged aspartyl residues continued in tissues completely lacking methyltransferase-mediated repair as animals aged beyond the limit set by the early deaths of the nontransgenic knockout mice. In addition to heart (Fig. 4) and

[3] When damaged aspartyl residues in brain proteins from *Pcmt1*−/− mice were quantitated as a function of time of base treatment, a linear increase of 1.8 pmol of damaged residues/mg of protein/min was obtained. This increase resulted from the creation of new damaged residues in the proteins. In contrast, quantitation of damaged residues in brain proteins from *Pcmt1*+/+ mice gave a biphasic increase with time of base treatment. For the first 5 min, the slope of the line was 33.4 pmol of damaged residues/mg of protein/min (largely reflecting new sites generated by hydrolysis of endogenous methyl esters), but for the next 355 min the slope was 1.8 pmol of damaged residues/mg of protein/min, reflecting base-catalyzed generation of new damaged sites as for the *Pcmt1*−/− proteins. It is thus possible to correct the data to quantitate endogenous methylated sites. We found that inclusion of endogenous methyl esters increases by 2.1-fold the damage estimate in extracts from wild-type brain. Because deesterification of a methylated L-isoaspartyl residue produces an unmethylated L-isoaspartyl residue only about 80% of the time, the true number of damaged residues in wild-type brain proteins can be as much as 2.4-fold higher than the number of residues detected in the absence of base treatment. Even with this increase, however, there are still about 4-fold more damaged residues in *Pcmt1*−/− brain proteins than in wild-type brain proteins.

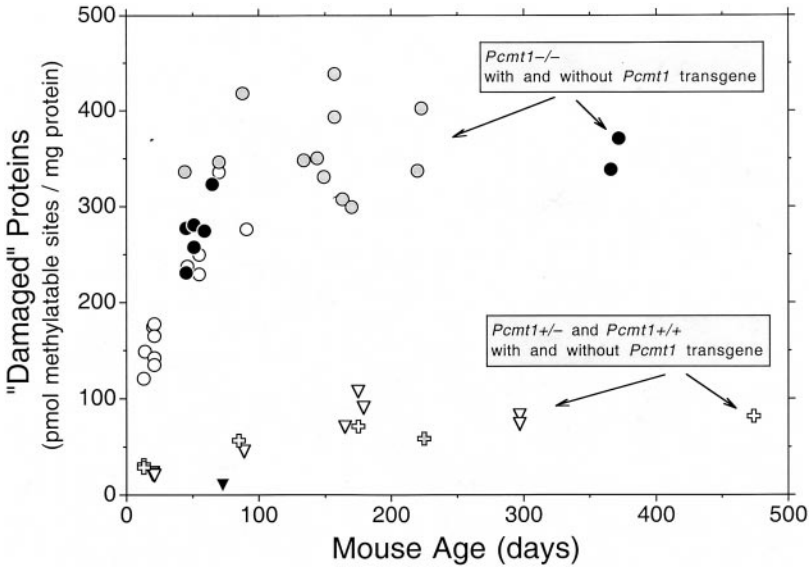

FIG. 4. **Accumulation of damaged aspartyl residues in heart cytosolic/microsomal polypeptides.** Levels of damage were assayed as described in Fig. 3. *Open circles*, *Pcmt1*−/− (n = 12); *gray circles*, transgenic *line 27 Pcmt1*−/− (n = 12); *black circles,* transgenic *line 29 Pcmt1*−/− (n = 8); *open crosses, Pcmt1*+/− (n = 6); *gray cross,* transgenic *line 27 Pcmt1*+/− (n = 1); *open triangles, Pcmt1*+/+ (n = 9); and *black triangle,* transgenic *line 29 Pcmt1*+/+ (n = 1).

TABLE II
Levels of damaged aspartyl residues in tissues of adult mice over 100 days in age

Recombinant human L-isoaspartyl (D-aspartyl) *O*-methyltransferase was used to quantitate damaged aspartyl residues in polypeptides remaining in the 20,800 × *g* supernatant after centrifugation of whole tissue homogenates as described under "Experimental Procedures." Values were first averaged from triplicate assays of individual tissue extracts and then averaged for the whole group of mice with standard deviation values. The number of animals used is given in parentheses. The error in each of the triplicate assays was generally very small (less than 5%).

Tissue	Genotype	
	Pcmt1−/− with transgene	*Pcmt1*+/− and *Pcmt1*+/+ with or without transgene
	pmol methylatable sites per mg protein	
Brain	829 ± 135 (21)	183 ± 29 (14)
Heart	356 ± 42 (11)	79 ± 14 (8)
Testis	500 ± 90 (13)	106 ± 56 (11)
Erythrocyte	77 ± 12 (20)	15 ± 2 (15)

TABLE III
Rates of accumulation of damaged aspartyl residues in Pcmt1−/− *mouse tissues*

The rates at which damaged residues accumulated nontransgenic *Pcmt1*−/− mouse tissues over the first 21 days after birth were calculated from the data in Figs. 3–6. It was assumed for these calculations that there are no damaged residues in newborn mice.

Tissue	Rate of accumulation
	pmol methylatable sites/mg protein/day
Brain	52
Testis	11.8
Heart	7.6
Liver	7.5
Erythrocyte	1.8

testis (Fig. 5), we also examined erythrocyte cytosol as a control (Fig. 6). Because erythrocytes are normally removed from the circulation after about 40 days (32), their average age in adult mice is constant, and older proteins cannot accumulate. Unexpectedly, the accumulation of damaged aspartyl residues in all of these tissues was quite similar. As in brain, the number of methylatable residues per mg protein increased only in animals younger than about 60 days and then leveled off as the mice got older (Figs. 4–6). The apparent plateau levels of damaged aspartyl residues in heart, testes, and erythrocytes from transgenic *Pcmt1*−/− mice averaged 4.5-, 4.7-, and 5.2-fold higher, respectively, than the average levels in tissues from mice expressing the endogenous methyltransferase, very similar to the 4.5-fold difference observed in brain. However, the absolute plateau level of damage in each tissue was significantly different, with brain having the highest and erythrocytes having the lowest levels in both the presence and in the absence of endogenous PCMT1 (Table II). As expected, the transgenic PCMT1 had no effect in tissues where it is not expressed.

The rates at which damaged residues in cytosolic proteins accumulated in young *Pcmt1*−/− mice have been calculated from the data in Figs. 3–6. These rates differ greatly between tissues, ranging from 51 pmol of methylatable residues/mg of protein/day in brains to 1.8 pmol of methylatable residues/mg of protein/day in erythrocytes (Table III). Because the rate at which aspartyl and asparaginyl residues arise in proteins should not decrease during the life of a mouse, we can use these rates to estimate the total number of damaged residues arising in adult mice. Assuming that a mouse is 15% protein by weight and that half of this protein is intracellular, a 20-g mouse would have 1.5 g of intracellular protein. If the average rate of damaged residue formation throughout the mouse is between 5 and 20 pmol/mg/day, as predicted from the values in Table III, there should be 7.5–30 nmol of newly damaged residues arising each day within the cells of an adult mouse.

Damaged Aspartyl Residues in Urine—The absence of increasing accumulation of damaged aspartyl residues in the cytosolic proteins of adult mice can result from repair of the damaged residues or from turnover of the proteins. Few peptidases cleave isoaspartyl bonds, but proteolysis of the surrounding residues creates isoaspartyl-containing dipeptides and tripeptides that can be excreted in the urine (33–36). Thus, if mice lacking endogenous PCMT1 do not have another repair pathway, they might excrete the damaged residues that are normally repaired in the cells of wild-type mice. To test this hypothesis, we examined urine from *Pcmt1*−/− and *Pcmt1*+/+ mice in several ways.

First, we directly quantitated urinary dipeptide levels after derivatization with *o*-phthalaldehyde and β-mercaptoethanol and separation by reverse-phase high pressure liquid chromatography. We did not detect significantly higher levels of isoaspartyl-containing dipeptides in urine from *Pcmt1* knockout

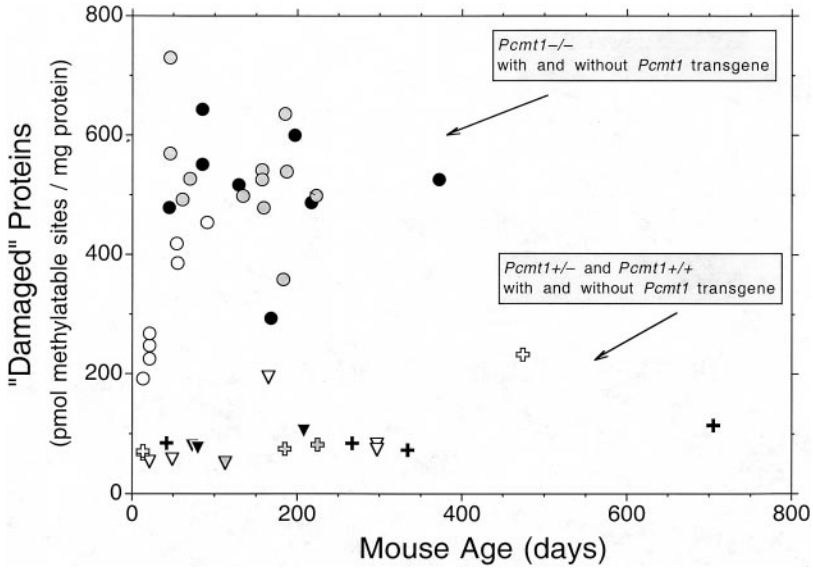

FIG. 5. **Accumulation of damaged aspartyl residues in testis cell cytosolic/microsomal polypeptides.** Levels of damage were assayed as described in Fig. 3. *Open circles*, *Pcmt1*−/−(*n* = 7); *gray circles*, transgenic *line 27 Pcmt1*−/− (*n* = 12); *black circles,* transgenic *line 29 Pcmt1*−/− (*n* = 8); *open crosses, Pcmt1*+/− (*n* = 3); *gray crosses,* transgenic *line 27 Pcmt1*+/− (*n* = 2); *black crosses,* transgenic *line 29 Pcmt1*+/− (*n* = 4); *open triangles, Pcmt1*+/+ (*n* = 5); *gray triangle,* transgenic *line 27 Pcmt1*+/+ (*n* = 1); and *black triangles,* transgenic *line 29 Pcmt1*+/+ (*n* = 3).

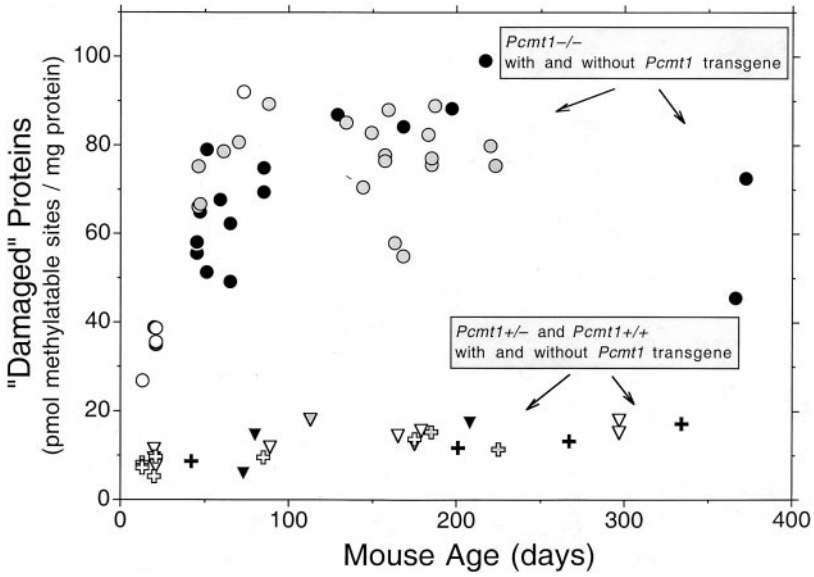

FIG. 6. **Accumulation of damaged aspartyl residues in erythrocyte cell cytosolic polypeptides.** Levels of damage were assayed as described in Fig. 3. *Open circles*, *Pcmt1*−/− (*n* = 8); *gray circles*, transgenic *line 27 Pcmt1*−/− (*n* = 20); *black circles,* transgenic *line 29 Pcmt1*−/− (*n* = 16); *open crosses, Pcmt1*+/−, (*n* = 8); *gray crosses,* transgenic *line 27 Pcmt1*+/− (*n* = 2); *black crosses,* transgenic *line 29 Pcmt1*+/− (*n* = 5); *open triangles, Pcmt1*+/+ (*n* = 9); *gray triangle,* transgenic *line 27 Pcmt1*+/+ (*n* = 1); and *black triangles,* transgenic *line 29 Pcmt1*+/+ (*n* = 3).

mice (data not shown). For example, isoaspartylglycine ("β-aspartylglycine"), the most abundant of these dipeptides in human urine (35), was present at 28.2 pmol/μg creatinine in urine from a 91-day-old *Pcmt1*−/− mouse and at 27.5 pmol/μg creatinine in urine from a 40-day-old wild-type mouse. These results suggest that much of this dipeptide might originate in dietary, extracellular, or unrepairable intracellular proteins rather than from the lack of repair in *Pcmt1*−/− cells.

Second, peptides with an N-terminal isoaspartyl residue might accumulate because the isopeptide bond is resistant to aminopeptidase activity. To determine whether more of these peptides accumulated in *Pcmt1*−/− than in *Pcmt1*+/+ mice, we compared the urinary levels of free amino acids and of amino acids released by acid hydrolysis, which cleaves both aspartyl and isoaspartyl bonds. Although wild-type urine contained 1.5-fold more total amino acid residues (free and peptide bound) than did knockout mouse urine, both urine samples contained 8–9-fold more peptide-bound residues than free residues (data not shown). This indicates that the knockout mice were not excreting elevated levels of peptides or proteins relative to wild-type mice.

Finally, we used the recombinant L-isoaspartyl/D-aspartyl methyltransferase as an analytical probe for L-isoaspartyl residues in peptides large enough to be methylated efficiently (tetrapeptides and larger) (15). Methylatable residues were found in all of the urine samples assayed, and the level of damage increased with the age of the mouse (Fig. 7). We observed no significant difference in the number of methylatable sites, relative to the amount of creatinine, in urine from *Pcmt1*−/− animals and from *Pcmt1*−/+ and *Pcmt1*+/+ controls in the 13–14- and 20–21-day-old groups (Fig. 7). However, urine from older *Pcmt1*−/− mice contained almost 2-fold more methylatable sites than urine from control animals (Fig. 7). Assuming that 435–783-day-old mice weighing 20 g excrete 0.67 mg of creatinine per day (37), then *Pcmt1*−/− mice excrete about 9.5 nmol and age-matched control animals about 5.1 nmol of methylatable L-isoaspartyl residues per day. Thus, at least 4.4 nmol of damaged residues that are repaired in wild-type mice are excreted each day by knockout mice that are not accumulating additional damaged residues within their cells. This daily excretion of damaged aspartyl residues in the urine represents 15–67% of the total daily production of intracellular aspartyl damage estimated above. This result indicates that proteolysis leading to urinary excretion is a significant factor in limiting the accumulation of damaged proteins in the absence, and perhaps as well in the presence, of the repair methyltransferase.

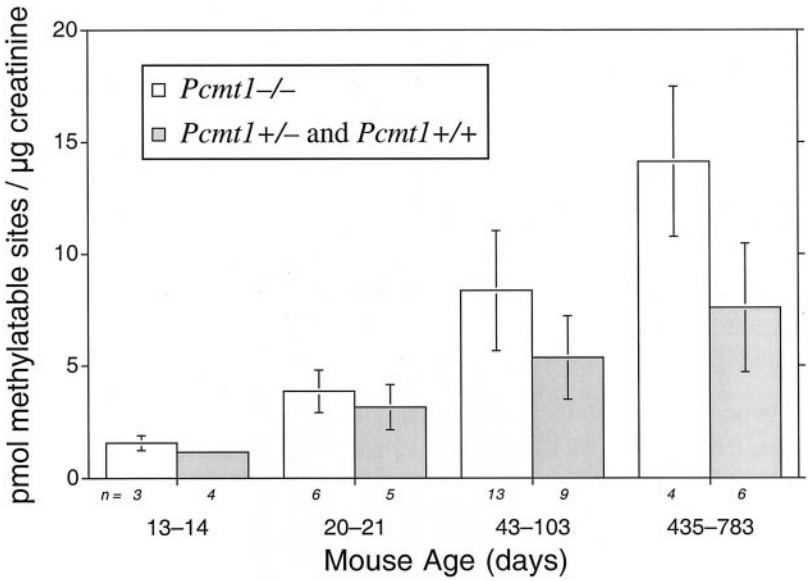

FIG. 7. **Damaged aspartyl residues in mouse urine that are recognized *in vitro* by the L-isoaspartyl (D-aspartyl) *O*-methyltransferase.** Each urine specimen was collected in a single voiding and was assayed without further treatment. Methylatable damaged residues were quantitated with recombinant human methyltransferase as described in Fig. 3. *Open bars,* urine from nontransgenic *Pcmt1*−/− mice; *shaded bars,* urine from nontransgenic *Pcmt1*+/− and *Pcmt1*+/+ mice. The difference between the values for these groups is statistically significant for the 43–103- and 435–783-day-old mice ($p = 0.006$ and 0.020, respectively) but not for the 13–14- or 20–21-day-old mice ($p = 0.161$ and 0.263, respectively).

DISCUSSION

We have been examining mice lacking PCMT1 to determine whether ineffective repair of damaged aspartyl and asparaginyl residues contributes to disease or to the deleterious effects of aging. We report here that damaged aspartyl residues in *Pcmt1*−/− brain proteins from 13 to 14-day-old mice were already eight times more abundant than those in aged-matched wild-type mice and accumulated at a rate of about 52 pmol/mg of protein/day until 20–21 days. Damaged residues continued to accumulate but at a lower rate, averaging 9 pmol/mg protein/day between days 21 and 55. The rate of accumulation appeared to decrease further in the brains of older *Pcmt1*−/− mice, but few of these mice live beyond this age. A similar pattern of damage accumulation has been recently observed by Shimizu *et al*. (38) in independently derived *Pcmt1*−/− mice. In contrast to the *Pcmt1*−/− mice, however, *Pcmt1*+/+ and *Pcmt1*+/− mice kept the level of damaged residues very low for at least 2 years. Although they had only about half of the methyltransferase activity, *Pcmt1*+/− mice survived as long as *Pcmt1*+/+ mice.

The high level of PCMT1 activity in wild-type brain, the fact that damaged residues rapidly accumulate in the brain in the absence of the methyltransferase, and the seizures in the *Pcmt1*−/− mice suggested that this enzyme plays a critical role in normal brain function. PCMT1, however, is expressed in all mammalian tissues and presumably is involved in repair throughout the body. We were therefore also interested in examining what would happen if tissues lacking this repair were allowed to accumulate higher levels of damage over several years.

To answer these questions, we performed a "brain rescue" experiment by creating mice with a *Pcmt1* transgene under the control of a neuron-specific promoter in a genetic background lacking the endogenous *Pcmt1* gene. Here, we obtained mice expressing the methyltransferase only in the brain. Although the expression of the transgene-derived methyltransferase activity in the brains of these mice was only 6.5–13% of the level in wild-type mice, the transgenic mice lived much longer and accumulated only half the damaged residues found in non-transgenic knockout animals. The success of this rescue experiment further supported the importance of the methyltransferase in the brain.

We reported previously (18) that mice lacking the repair methyltransferase are more sensitive to the seizure-inducing drug metrazol than are wild-type mice and that the anti-seizure drugs valproic acid and clonazepam prolonged the life span of these animals (20). It is likely that, by decreasing the accumulation of damaged aspartyl residues, the transgene-derived methyltransferase raised the seizure threshold of the rescued mice to an intermediate level between the thresholds of nontransgenic *Pcmt1*−/− and wild-type mice. Immunohistochemical studies revealed that the transgene-derived methyltransferase was expressed largely in neurons, in both line 27 and line 29. However, the pattern of neuronal expression was distinct in the two lines.[4] These findings indicate that the expression of *Pcmt1* in neurons (rather than glia) is paramount in the prevention of the fatal seizure disorder. At this point, however, we do not know whether the methyltransferase is critical for the function of all neurons or only for certain types of neurons.

Because many of the transgenic *Pcmt1*−/− mice lived for several hundred days, we were able to examine the long term accumulation of damaged residues in peripheral organs, where the methyltransferase is not expressed at all. We found no apparent defects in these mice, and initial pathological studies were unremarkable. We found that damaged aspartyl residues accumulated in an age-dependent manner only in relatively young mice and attained plateau levels by 100 days of age. At first approximation, the rate at which damaged residues arise in proteins remains constant with time; thus, the plateau levels of damage in tissues of older mice must result from processes of repair and/or turnover of these residues. We have been especially interested in the situation in mouse tissues that lack the repair methyltransferase, where the levels of damage are much greater but which still approach plateau values with age. We suggest that enhanced proteolytic degradation may limit the accumulation of proteins containing damaged aspartyl residues, especially in methyltransferase-deficient tissues. Clear evidence has been presented for the selective proteolytic degradation of spontaneously damaged calmodulin in both HeLa cells (6) and in *Xenopus* oocytes (39).

Isoaspartyl linkages themselves are generally not cleaved by mammalian proteases (33, 34). We have thus investigated the possibility that proteolysis of proteins containing damaged aspartyl residues in PCMT1-deficient mice may be reflected in the increased urinary output of peptides containing L-isoaspartyl residues. In fact, damaged aspartyl residues in proteins fed

[4] C. Farrar, E. Kim, S. Young, S. Clarke, and C. Houser, unpublished data.

to rats are excreted as isoaspartyl-containing dipeptides in the urine (40), and it has been proposed that at least some endogenous isoaspartyl residues are dealt with in a similar manner (41). Furthermore, an L-isoaspartyl residue that arises in collagen has been found in human urine as part of an eight-residue peptide (42). We found that urine from *Pcmt1*−/− mice contains more peptides with damaged aspartyl residues than urine from *Pcmt1*+/+ mouse urine. Because PCMT1 repairs intracellular proteins (14), our results provide the first evidence that damaged aspartyl residues that arise within cells can be excreted in the urine.

Is the elevated level of altered aspartyl residues in the urine from *Pcmt1*−/− mice enough to account for the observed steady state level of damaged proteins in tissues despite continuing spontaneous generation of isoaspartyl residues? From the rates of damage accumulation measured in tissues of young *Pcmt1*−/− mice, we estimated that 7.5–30 nmol of newly damaged aspartyl residues are generated each day. The daily excretion of damaged residues in the urine of *Pcmt1*−/− mice was 4.4 nmol more than that in wild-type mice, demonstrating that a significant fraction of the damage can be metabolized by proteolysis. Additional isoaspartyl peptides may be present in the urine that are not readily recognized by the methyltransferase (15), although we did not detect increased levels of generally poorly recognized dipeptides and N-terminal L-isoaspartyl peptides.

Interestingly, the ability of the proteolytic pathway to stem the accumulation of damaged aspartyl residues is apparently insufficient to prevent seizures in animals lacking the repair methyltransferase. Thus, the repair methyltransferase is needed (at least in the brain) to lower the level of damaged residues beyond what the degradation mechanisms can accomplish. Furthermore, it is possible that proteolysis of proteins that contain covalent modifications important to learning and memory could have undesirable effects in that their replacement proteins would not be appropriately modified (43).

Unlike the brain, other tissues appear to be able to function relatively normally with higher levels of damaged aspartyl residues. The importance of the methyltransferase in maintaining a lower steady state level of damaged aspartyl residues in these tissues remains to be determined. It is certainly possible that mice reared under other conditions (*e.g.* outside of a vivarium) would be more susceptible to pathologies in the absence of the repair methyltransferase.

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