Influence of genetic knockout of Pept2 on the in vivo disposition of endogenous and exogenous carnosine in wild-type and Pept2 null mice

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Submitted 3 September 2008; accepted in final form 12 February 2009


Carnosine, a dipeptide and a biological antioxidant, is widely distributed in many tissues, including blood, brain, and kidney. The amino acid composition of carnosine, an α-amino group and L-conformation, makes it an effective antioxidant. Using a genetic knockout model of Pept2, the authors examined the disposition of carnosine in wild-type and Pept2 null mice. The results demonstrate that Pept2 plays an important role in the uptake of carnosine in vivo. Furthermore, the results highlight the potential therapeutic benefits of carnosine, particularly in the context of renal failure and diabetic nephropathy.

AN ENDOGENOUS DIPEPTIDE, β-alanyl-L-histidine (carnosine), is abundantly expressed in the skeletal muscle and central nervous system (6, 31). Carnosine has many physiological roles, including its action as a cytosolic buffer (21), neurotransmitter/neuromodulator (2), and metabolic reservoir of histidine, which is converted into histamine during physiological stress in mammals (6). The dipeptide also possesses strong antioxidant and free radical scavenging activities (9, 21), and, as a result, carnosine is taken exogenously as a dietary supplement. More recently, protective effects of carnosine have been demonstrated in rodent models of global and focal cerebral ischemia (22, 29), in rats with ischemia/reperfusion-induced acute renal failure (7, 14), and in patients with diabetic nephropathy (12). Having favorable structural attributes, such as a β-amino group and L-conformation, the dipeptide is a substrate of Pept2 (30), a member of the proton-coupled oligopeptide transporter family SLC15A.

Pept2 is primarily localized in the apical membrane of kidney epithelial cells, with immunolocalization studies specifically identifying the transporter in S2 and S3 segments of the proximal tubule (28). In brain, Pept2 is expressed at the apical side of choroid plexus epithelial cells of the blood-cerebrospinal fluid (CSF) barrier (1), and in astrocytes (newborns and neuronal cells (newborn and adults) of brain parenchyma (26). Pept2 protein has also been identified in the alveolar lining of lung tissue and mammary glands, as well as in retina and spleen (5). The primary physiological roles of Pept2 include the reabsorption of peptides from glomerular filtrate in renal proximal tubules, the maintenance of brain homeostasis by controlling peptide trafficking in brain interstitial fluid and peptide removal from CSF, and the facilitation of peptide uptake for action by intracellular peptidases. Still, the significance and interplay of these physiological roles in vivo are not entirely clear, particularly for an endogenous substrate, such as carnosine, where there is both production (carnosine synthetase) and degradation (carnosinase).

A previous study has demonstrated that carnosine is taken up into rat choroid plexus primary cell cultures and mouse choroid plexus whole tissue by Pept2 (31). However, the significance of Pept2 in mediating the disposition of this dipeptide in vivo has not been investigated. It is hypothesized that Pept2 ablation will have a profound impact on the systemic pharmacokinetics of carnosine, as well as on the regional exposure of carnosine in kidney and brain. Differences in Pept2-mediated disposition may also alter the physiological and pharmacological benefits of carnosine, including its renal (7, 14) and neuroprotective (22, 29) effects after ischemic insult.

The current study will determine the physiological in vivo levels of carnosine in Pept2-deficient mice. Moreover, this study will examine the influence of Pept2 on the pharmacokinetics of exogenously-administered carnosine.

MATERIALS AND METHODS

Chemicals. [3H]Carnosine (sp act: 7.0 Ci/mmol), [3H]histidine (sp act: 44 Ci/mmol), and [14C]dextran-MW 70,000 (sp act: 79 mCi/mmol) were purchased from Moravek Radiopharmaceuticals (Brea, CA). Unlabeled carnosine was obtained from Sigma-Aldrich (St. Louis, MO). Cytoscint scintillation fluid and hyamine hydroxide were

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obtained from ICN Biomedicals (Irvine, CA). All other chemicals were obtained from standard sources.

Animals. Gender- and weight-matched wild-type (Pept2+/+) and null (Pept2−/−) mice (>99% C57BL/6 genetic background) 8 to 10 wk of age were generated in-house (27) and used for all study designs. Animals were housed in a temperature-controlled environment with a 12:12-h light-dark cycle and given ad libitum access to food and water. All experiments with mice were performed in accordance with the guidelines from the National Institutes of Health for the care and use of animals, and were approved by the Institutional Animal Care and Use Committee of the University of Michigan, Ann Arbor, Michigan.

Endogenous tissue levels of carnosine. Following pentobarbital sodium anesthesia (65 mg/kg ip), a blood sample was obtained from wild-type and Pept2 null mice, and the plasma harvested. A CSF sample (~5 μl) was then taken from the cisterna magna. After decapitation, tissue samples were immediately obtained from the choroid plexuses (lateral and fourth ventricles), cerebral cortex and olfactory bulb, along with skeletal muscle, spleen, and kidney. Most tissues were homogenized with water to make a 5% homogenate and then extracted with four volumes of 75% methanol-25% boric acid after vortex-mixing for 2 min, sonication for 20 min, and centrifuging at 15,000 g for 10 min. Choroid plexus tissue was prepared as a 1% homogenate followed by extraction with one volume of the methanol-boric acid mixture. CSF and plasma samples were added directly to six volumes of the methanol-boric acid mixture. Supernatants were then stored at −20°C until subsequent analysis.

After thawing, the supernatants were diluted (2- to 5-fold) with boric acid buffer (0.1 M boric acid, 0.1 M potassium chloride, 0.1 M sodium hydroxide, adjusted to pH 9.9 with 4 M hydrochloric acid). A 100-μl aliquot of the diluted supernatant was then derivatized for 5 min with 100 μl of OPA reagent (2.0 ml of 5.0 mg/ml phthaldialdehyde-515TR Series Flow Scintillation Analyzer; PerkinElmer Life and Analytical Sciences, Boston, MA) by using an anisotropic hydrophilic membrane that excluded molecules greater than 30 kDa. The device was capped, equilibrated for 15 min at 37°C in a 35-degree fixed angle rotor, and centrifuged at 1,800 g for 25 min at 37°C. The protein-free ultrafiltrate was then collected for each sample. The unbound fraction in plasma was calculated as the ratio of supernatant to total plasma. Carnosine concentrations were determined by HPLC consisting of a Waters C18 column (5 μm, 4.6 × 250 mm) at a flow rate of 1.0 ml/min under ambient conditions. The mobile phase consisted of 75% solvent A (10% methanol in 0.05 M sodium acetate, pH 5.5) and 25% solvent B (80% methanol in 0.05 M sodium acetate, pH 5.5), with a gradient elution running at the following: 0–10 min (75% solvent A), 10–25 min (70% solvent A), 25–30 min (65% solvent A), 30–32 min (55% solvent A), 32–35 min (10% solvent A), and 35–42 min (75% solvent A). Under these conditions, carnosine eluted at 25.5 min, as confirmed with authentic sample.

Standard curves were prepared with diluted olfactory bulb extracts over the carnosine concentration range of 0.054–5.4 μM. The peak area vs. concentration relationships were linear (weighted 1/C2) in which r2 values were ≥ 0.999 (n = 6). Accuracy of the assay was determined after correcting for endogenous concentrations of carnosine [i.e., %recovery = 100 × (total concentration − endogenous concentration)/spiked concentration] and was 102.3% with a coefficient of variation ≤ 5.3%. The intraday and interday precisions of the assay were ≤ 4.9%. The extracted samples were stable for 60 days at −20°C.

Systemic pharmacokinetic and tissue distribution studies. Pept2−/− and Pept2−/− null mice were anesthetized with pentobarbital sodium (65 mg/kg ip). [1H]Carnosine was injected into the tail vein of mice as a single bolus injection (1 nmol/g body wt; 5 μl/g in normal saline). Serial blood samples (~20 μl, via tail clipping) were collected at 0, 0.25, 1, 2, 5, 10, 20, and 30 min, placed in 0.2-ml thin-wall PCR tubes (United Laboratory Plastics; St. Louis, MO) containing 1 μl of 7.5% potassium EDTA, and centrifuged at 3,000 g for 3 min at room temperature. A 5-μl aliquot of plasma supernatant was collected for each sample, mixed with 6 ml of scintillation fluid, vortex-mixed for 5 s, and then allowed to stand for 24 h at ambient temperature. Radioactivity (measured in dpm/ml) for each plasma sample was measured by a dual-channel liquid scintillation counter (model LS 3801; Beckman Coulter, Fullerton, CA).

An intravenous bolus of [14C]dextran-MW 70,000 (1 μCi/mouse) was administered 2 min prior to harvesting the 30-min tissue samples to correct for the vascular space (25). A skin incision was made in the dorsal neck region to allow insertion of a 30-gauge needle into the cisterna magna for CSF sampling (~5–10 μl). The mouse was immediately decapitated and a 10-μl blood sample was obtained. Various organs/tissues were harvested at this time, including the cerebral cortex, olfactory bulb, combined lateral and fourth ventricle choroid plexuses, kidney, liver, eye, lung, spleen, small and large intestines, and skeletal muscle. The tissue samples were blotted dry and weighed, solubilized in 1 M hyamine hydroxide, and then incubated for 48 h at 37°C. Solubilized tissue samples (and CSF) were mixed with 6 ml of scintillation fluid and left to stand for 24 h at room temperature. Radioactivity (measured in dpm/ml) in the blood, CSF, and tissue samples was measured by a dual-channel liquid scintillation counter.

Renal clearance studies. Following pentobarbital sodium anesthesia (65 mg/kg ip), Pept2−/− and Pept2−/− mice were administered [1H]carnosine (1 nmol/g body wt; 5 μl/g in normal saline) by tail vein injection. Blood samples (~20 μl, via tail clipping) were collected serially over 30 min and the plasma harvested. The total urine of each animal was aspirated directly from the bladder with a 28G/1 U-100 insulin syringe at 30 min. Radioactivity in the plasma and urine was determined by dual-channel liquid scintillation counting.

Plasma protein binding studies. The protein binding of carnosine was determined by an ultrafiltration method (20) with minor modification. Blank plasma from each genotype was spiked with unlabeled and radiolabeled carnosine (0.1 μCi/ml) to produce concentrations of 0.1, 1, and 10 μM, values that represent the plasma concentration range observed after a 1 nmol/g intravenous dose of carnosine. A 0.5-ml aliquot of each standard was added to a disposable Microcon YMT-30 centrifugal filter device (Millipore, Billerica, MA) by using an anisotropic hydrophilic membrane that excluded molecules greater than 30 kDa. The device was capped, equilibrated for 15 min at 37°C in a 35-degree fixed angle rotor, and centrifuged at 1,800 g for 25 min at 37°C. The protein-free ultrafiltrate was then collected for each sample. The unbound fraction in plasma was calculated as the ratio of carnosine concentration in the ultrafiltrate to that in the original plasma sample. Liquid scintillation counting was used to determine radioactive counts in the samples.

Stability studies. [1H]Carnosine and [1H]histidine peaks were detected using an HPLC system, a Supelco C18 column (5 μm, 4.6 × 25 cm) (Sigma-Aldrich), and a radiochemical detector (FLO-ONE 515TR Series Flow Scintillation Analyzer; PerkinElmer Life and Analytical Sciences, Boston, MA) as described previously (31). The mobile phase comprised 0.10 M NaH2PO4 buffer (pH = 3.2) and 0.10% heptafluorobutyric acid, pumped isocratically at 1.0 ml/min under ambient conditions. Under these conditions, histidine and carnosine eluted at 5.5 min and 9.1 min, respectively, as confirmed by authentic samples.

The metabolic stability of [1H]carnosine was determined in plasma, kidney, and urine samples following an intravenous dose (1 nmol/g body wt) in wild-type and Pept2 null mice. Blood samples (100 μl) were collected by cardiac puncture at 2, 5, 10, 20, and 30 min, and the plasma was harvested. Kidney samples (200 mg) were obtained at 30 min and homogenized in 1 ml water (4°C). A 0.2-ml volume of trichloroacetic acid (10% wt/vol) was added to one volume of plasma or kidney homogenate, vortex-mixed for 1 min, and then centrifuged at 15,000 g for 10 min at room temperature. Urine samples were also centrifuged at 15,000 g for 10 min (ambient conditions) to remove any particulates. Resultant supernatants were injected into the HPLC, and stability was evaluated by the ratio of carnosine area to the total area of carnosine and histidine (×100 for percent). The physicochemical
integrity of \[^{[3]H}\]carnosine stock solution (1 \(\mu\)Ci/ml) was also determined at 25°C and 37°C following 0.5- and 24-h incubations.

Data analysis of carnosine systemic pharmacokinetics. The plasma concentration-time profiles of carnosine displayed bieponential pharmacokinetics and were best described by a two-compartment open model with first-order elimination and uniform weighting (WinNonLin version 5.1; Pharsight, Mountain View, CA). The model goodness-of-fit was determined by evaluating the coefficient of determination (\(\rho^2\)), the coefficient of variation of parameter estimates, and by visual inspection of the residuals. Pharmacokinetic parameters included area under the plasma concentration-time curve (AUC), total systemic clearance (CL), volume of the central compartment (V1), volume of distribution steady state (Vdss), terminal half-life (\(t_{1/2}\)), and mean residence time (MRT).

Data analysis of carnosine renal pharmacokinetics. The renal clearance (CLR) of carnosine was calculated as: CLR = \(\text{CL}_{\text{R}} = \frac{\text{Ae}}{\text{fu} \times \text{GFR}}\) (15), where fu is the fraction of carnosine unbound in plasma, GFR is the glomerular filtration rate (a measure of functional nephron mass), and \(\text{CL}_{\text{R}}\) is the available fraction reabsorbed by PEPT2. The renal clearance equation can be transformed to (25): CLR = \(\frac{\text{Ae}_{30}}{\text{AUC}_{30}}\), where \(\text{Ae}_{30}\) is the amount of carnosine excreted unchanged in the urine at 30 min and \(\text{AUC}_{30}\) is the AUC from 0–30 min (determined noncompartmentally by partial areas using WinNonLin). In the absence of tubular secretion (of which there is no evidence for this dipeptide), the renal clearance of carnosine can be expressed as (15): CLR = \(\text{CL}_{\text{R}} = \frac{\text{fu} \times \text{GFR}}{1 - \text{F}}\) where \(\text{fu}\) is the fraction of carnosine unbound in plasma, GFR is the glomerular filtration rate (a measure of functional nephron mass), and \(\text{F}\) is the fraction of available dipeptide that is reabsorbed from tubular fluid. The excretion ratio (ER) and fraction reabsorbed (F) was determined according to the following: ER = \(\frac{\text{CL}_{\text{R}}}{\text{fu} \times \text{GFR}}\) = 1-F. Based on the sequential expression of PEPT1 and PEPT2 in the proximal tubule of the nephron (28), the renal clearance equation can be transformed to (25): CLR = \(\text{CL}_{\text{R}} = \frac{\text{fu} \times \text{GFR} \times (1-F)}{1-(1-F)}\), where \(F\) is the available fraction of carnosine reabsorbed by PEPT1 and \(F\) is the available fraction reabsorbed by PEPT2. Since \(F = 0\) in PEPT2 \(^{-/-}\) mice, an estimate of \(F\) can be made in these mice. An estimate of \(F\) can then be made in PEPT2 \(^{+/+}\) mice with the assumption that \(F\) is unchanged in wild-type animals (11, 20, 24). The relative contribution of each transporter to the reabsorption of carnosine can then be calculated as % PEPT1 = 100 \(\times \frac{F_{1}}{F_{1}+F_{2}}\) and % PEPT2 = 100 \(\times \frac{F_{2}}{F_{1}+F_{2}}\).

Statistics. The data are reported as means ± SE. Statistical comparisons between the two genotypes were performed using an unpaired t-test (Prism v4.0; GraphPad Software, San Diego, CA).

RESULTS

Endogenous levels of carnosine in tissue and biological fluid. The endogenous concentrations of carnosine in plasma, CSF, and selected organs of wild-type and Pept2 knockout mice are shown in Table 1. Plasma levels of carnosine were very low (<3 \(\mu\)M) and not significantly different between the two genotypes. In wild-type animals, the tissue-to-plasma concentration ratios of carnosine ranged from 12 (spleen) to 716 (olfactory bulb). The CSF/plasma ratio was 3.4, indicating that carnosine was being synthesized in brain. Tissue levels of carnosine in Pept2 null mice, compared with wild-type mice, were significantly lower in choroid plexus (\(-85–90\%)\), olfactory bulb (\(-40\%)\), and spleen (\(-35\%)\). Although carnosine levels were lower in kidney (\(-30\%)\) and cerebral cortex (\(-15\%)\), the changes did not reach statistical significance. Surprisingly, the concentrations of carnosine in CSF were not different between genotypes, and the skeletal muscle levels were significantly higher (\(-50\%)\) in Pept2 null mice. Given that this dipeptide may be taken as a dietary supplement, it was also important to determine whether the pharmacokinetics, systemic exposure, and tissue distribution of carnosine differed between wild-type and Pept2 knockout mice when administered exogenously.

Systemic pharmacokinetics and tissue distribution of exogenous carnosine. As shown in Fig. 1, the plasma concentrations of exogenous carnosine were significantly lower in Pept2 \(^{-/-}\) mice compared with Pept2 \(^{+/+}\) animals, and in the range of physiological levels for endogenous carnosine. The altered plasma profiles are reflected in the pharmacokinetic parameters shown in Table 2. The systemic clearance (CL) of null animals is twofold higher compared with wild-type animals (\(P < 0.001\)), resulting in a twofold lower systemic exposure (AUC) (\(P < 0.01\)). PEPT2 had no effect on volume of distribution in the central compartment (V1); however, the steady-state volume (Vdss) was somewhat higher in null mice (\(P < 0.05\)). In contrast, no significant differences were observed in the terminal half-life (\(t_{1/2}\)) and mean residence time (MRT) of carnosine. These last two parameters are not statistically different since they reflect changes in both distribution and elimination (which increase in Pept2 \(^{-/-}\)). Fig. 2 shows the tissue concentrations of carnosine, normalized for plasma concentrations, 30 min following an intravenous bolus dose of dipeptide. PEPT2 ablation had a significant effect on the ability of many tissues to accumulate carnosine. Most notably, lower tissue/plasma concentration ratios of carnosine were observed in the kidney (6-fold), choroid plexus (8-fold), spleen (12-fold), eye (3-fold), lung (3-fold), cerebral cortex (3-fold), olfactory bulb (3-fold), and muscle (2-fold) of Pept2 knockout mice. In contrast to these results, PEPT2 \(^{-/-}\) mice had an eightfold higher CSF/plasma concentration ratio (\(P < 0.001\)).

Renal clearance of exogenous carnosine. An analysis of the renal tubular handling of carnosine is shown in Table 3 and Fig. 3. The renal clearance of carnosine (CLR) was \(17\)-fold higher in the Pept2 null mice compared with wild-type mice (\(P < 0.001\)), resulting in a significantly higher fraction of dipeptide being excreted unchanged in the urine at 30 min (fe\(^{\text{ex}}\) (\(P < 0.001\)). Ultrafiltration studies showed no protein binding of carnosine across the relevant plasma concentrations of 0.1–10 \(\mu\)M, and, as such, the fraction unbound (fu) for carnosine in plasma was unity. GFR was fixed at 250 \(\mu\)l/min based on the consistent values between genotypes in two previous studies by our group in gender-matched mice of similar age and weight (20, 25). Since the excretion ratio (ER) of carnosine represents its renal clearance, corrected for filtration clearance (fu×GFR), the significantly higher ER in null mice reflects the reduced reabsorption of carnosine in Pept2-deficient mice (i.e., \(F = 0.94\) vs. 0.19 for wild-type and null mice, respectively). Moreover, of the two oligopeptide transporters expressed in kidney, PEPT2 was responsible for the

<table>
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<tr>
<th>Tissue or Biological Fluid</th>
<th>Pept2 (^{-/-})</th>
<th>Pept2 (^{+/+})</th>
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<tbody>
<tr>
<td>Plasma</td>
<td>0.0025±0.0004</td>
<td>0.0028±0.0003</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>0.0086±0.0007</td>
<td>0.0089±0.0010</td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>0.20±0.02</td>
<td>0.206±0.010*</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>1.79±0.05</td>
<td>1.12±0.089</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.075±0.006</td>
<td>0.065±0.007</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.029±0.005</td>
<td>0.019±0.002*</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.048±0.010</td>
<td>0.034±0.003</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1.14±0.14</td>
<td>1.70±0.21*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE (\(n = 4–7\)). Values are expressed as mmol/kg or mmol/l *\(P < 0.05\) and †\(P < 0.01\), as compared with wild-type mice.
great majority of dipeptide reabsorption. In this regard, PEPT1 accounted for only 17% of carnosine’s reabsorption from tubular fluid, while PEPT2 accounted for 83% of dipeptide reabsorption in the kidney.

Stability of exogenous carnosine. The physicochemical integrity of [3H]carnosine stock solutions was maintained during 0.5- and 24-h incubations, at ambient temperature and 37°C (data not shown). While the 30-min urine collections of both genotypes were stable (<5% degradation), ~12% of carnosine was degraded in the 30-min kidney samples of wild-type, but not Pept2 null, mice (Fig. 4). Likewise, serial plasma samples from Pept2−/− mice were stable over 30 min (<3% degradation), as were the 2-, 5-, and 10-min plasma samples from Pept2+/+ mice (<10% degradation). However, the 20- and 30-min plasma samples showed 14.7% and 20.2% degradation, respectively, in wild-type mice. As a result, the plasma concentrations of carnosine in Pept2+/+ mice were corrected for the degradation observed at these times.

DISCUSSION

Studies using wild-type and knockout mice offer a unique opportunity to study the role and relevance of a particular protein under physiological in vivo conditions. In this study, we evaluated the disposition of endogenous carnosine levels in wild-type and Pept2 null mice as well as the disposition of carnosine when administered exogenously to these genotypes. The latter studies (i.e., of exogenous carnosine) were easier to interpret since differences between the two genotypes reflect the loss of PEPT2. In these studies, we found that PEPT2 was the major oligopeptide transporter responsible for dipeptide reabsorption in the kidney, that the regional effects of PEPT2 in several organs, including the brain (e.g., choroid plexus and CSF), were greater than the systemic effects on dipeptide exposure, and that transport-metabolic coupling of dipeptide occurs to retain amino nitrogen. The former studies were more difficult to interpret since, in addition to transport and metabolism, endogenous levels of carnosine would also reflect the rate of dipeptide production/synthesis.

In comparing the endogenous and exogenous levels of carnosine between wild-type and Pept2 null mice, several important differences were observed. First, endogenous carnosine levels in skeletal muscle were 50% higher in null compared with wild-type mice, whereas exogenous levels of carnosine were 40% lower in the skeletal muscle of null animals. Second, endogenous carnosine levels were reduced to a lesser extent than exogenous levels of dipeptide in the spleen, kidney, cerebral cortex, and olfactory bulb of null vs. wild-type mice.

Table 3. Renal pharmacokinetics of carnosine in wild-type and Pept2 knockout mice after a 1 nmol/g intravenous bolus dose

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pept2+/+</th>
<th>Pept2−/−</th>
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<tbody>
<tr>
<td>CL, μl/min</td>
<td>11.5±3.3</td>
<td>262±16;‡</td>
</tr>
<tr>
<td>GFR, μl/min</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>fu</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>fe30</td>
<td>0.023±0.005</td>
<td>0.50±0.03;‡</td>
</tr>
<tr>
<td>ER</td>
<td>0.06±0.02</td>
<td>0.81±0.08;‡</td>
</tr>
<tr>
<td>F</td>
<td>0.94±0.02</td>
<td>0.19±0.08;‡</td>
</tr>
<tr>
<td>F1</td>
<td>0.19±0.08</td>
<td>0.19±0.08</td>
</tr>
<tr>
<td>F2</td>
<td>0.92±0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>%PEPT1</td>
<td>16.9±8.1</td>
<td>100</td>
</tr>
<tr>
<td>%PEPT2</td>
<td>83.1±7.2</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE (n = 7 mice). CL, renal clearance; GFR, glomerular filtration rate (values taken from Refs. 20, 25); fu, fraction unbound in plasma; fe30, fraction excreted unchanged in the urine over 30 min; ER, excretion ratio; F, fraction of available dipeptide reabsorbed; F1, fraction of available dipeptide reabsorbed by PEPT1; F2, fraction of available dipeptide reabsorbed by PEPT2; %PEPT1, %reabsorbed carnosine that occurs via PEPT1; and %PEPT2, %reabsorbed carnosine that occurs via PEPT2. †P < 0.05; ‡P < 0.01; ††P < 0.001.
The concentrations of [3H]carnosine in CSF were similar to that of all three compounds. It is interesting that in third, both the plasma and CSF levels of endogenous carnosine were unchanged in the two genotypes. In contrast, plasma levels of exogenous carnosine were about twofold lower, and CSF-to-plasma ratios of exogenous carnosine were about eightfold higher in the null mice. Although speculative, our findings suggest that under physiologic conditions, Pept2 knockout mice had a greater production/synthesis of carnosine to maintain homeostasis in both the systemic (i.e., plasma) and central (i.e., CSF) concentrations of endogenous dipeptide. Moreover, it is possible that the skeletal muscle and olfactory bulb were responsible for this greater production/synthesis rate given the magnitude of endogenous carnosine and carnosine synthetase in these tissues (18, 19). These adaptations would be important in regulating the neurotransmitter/neuromodulator actions of carnosine in the olfactory pathway (2) and in modulating the cytosolic buffering capabilities of carnosine in the muscle, as well as its antioxidant and free radical scavenging properties in the body (21).

PEPT2 ablation led to substantially lower levels of exogenous carnosine in tissues where the peptide transporter is predominantly expressed (e.g., cerebral cortex, olfactory bulb, choroid plexus, CSF, kidney, eye, lung, spleen, and skeletal muscle) (5, 23, 26). Given the very low concentrations of endogenous carnosine in plasma (<3 μM), compared with K_m = 50 μM for apical carnosine transport in SKPT cells expressing PEPT2 (13) and K_m = 60 μM for carnosine metabolism by purified mouse kidney carnosinase (17), it is very unlikely that these concentrations would affect the disposition of exogenous carnosine. Our current findings with exogenous carnosine are consistent with previous studies in which the in vivo disposition of glycylysarcosine (20) and cefadroxil (25) was examined in wild-type and Pept2 null mice. In particular, the CSF/blood ratios were 4–8 times higher in null vs. wild-type animals for all three compounds. It is interesting that in Pept2 null mice, the concentrations of [3H]carnosine in CSF were similar to that in plasma by 30 min. In contrast to the role of PEPT2 in clearing carnosine from CSF (as demonstrated here), the mechanisms by which carnosine enters the CSF from blood are still unknown, including any potential involvement of other peptide transporters. PEPT2 was largely responsible for the renal handling of glycylysarcosine, cefadroxil, and carnosine, accounting for 83–95% of their tubular reabsorption in kidney. With respect to pharmacokinetics, Pept2 null mice had systemic clearances that were 1.7–3.1 times higher than wild-type animals for all three compounds. A small but significant change was observed for the volume of distribution steady state of carnosine (38% increase), compared with nonsignificant changes for glycylysarcosine (21% increase) and cefadroxil (28% increase). Although the half-life of carnosine was reduced by 15%, the change was not statistically significant. In contrast, significant reductions in half-life were observed for both glycylysarcosine and cefadroxil (by about 35%). However, the greatest difference between genotypes occurred for carnosine renal clearance, which was about 18-fold larger in Pept2 null mice. In comparison, two- to threefold changes were observed between wild-type and knockout mice for the renal clearances of glycylysarcosine and cefadroxil. As demonstrated in SKPT cells, carnosine is expected to have a substantial cellular accumulation in kidney but minimal tubular reabsorption to blood because of its high influx clearance across apical membranes by PEPT2 and very low efflux clearance across basolateral membranes (13). In the absence of PEPT2, the kidney/plasma concentration ratio of carnosine was reduced sixfold.

Carnosine is hydrolyzed to its constituent amino acids by carnosinase (8, 17), a nonspecific dipeptidase with high enzymatic activity in the cytosol of proximal tubule renal epithelia (17). Although C57BL/6 mice express low levels of carnosinase in their kidneys (16), some instability of carnosine was still observed in our mice. In particular, after only 30 min, ~20% and 12% of the plasma and kidney samples of wild-type mice, respectively, were in the form of carnosine hydrolysis products. However, in Pept2 null mice, these same samples displayed little instability (~<6% degradation) as did the urine samples for both genotypes (~<5% degradation). These results can be explained by the greater PEPT2-mediated uptake, and subsequent renal hydrolysis, of carnosine in wild-type animals. Although other tissues may contribute to carnosine hydrolysis, this outcome is less likely, given the kidney’s unique combination of high PEPT2 (20, 25) and carnosinase activity (8). Amino acid transporters on the basolateral membrane of the renal epithelia (3) can then reabsorb the degradation products (i.e., β-alanine and/or L-histidine) back into plasma. Amino acid transporters on the apical membrane of renal epithelia (3) can efficiently reabsorb the degradation products of carnosine in renal filtrate, and, thereby, minimize the extent of hydrolysis products found in the urine of both wild-type and Pept2 null mice. The concerted transport-metabolic coupling of carnosine by PEPT2 and peptidases has been depicted for dipeptides.

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Fig. 3. Renal clearance of carnosine in Pept2^{+/+} and Pept2^{-/-} mice after a 1-nmol/g intravenous bolus dose (means ± SE, n = 7). The estimated GFR of 250 μl/min is indicated by a dashed line. ***P < 0.001.

Fig. 4. Stability of carnosine in plasma, kidney, and urine samples from Pept2^{+/+} and Pept2^{-/-} mice, 30 min after a 1-nmol/g intravenous bolus dose (means ± SE, n = 4). *P < 0.05; **P < 0.01.
previously in kidney (4) and was demonstrated previously for glycylglutamine in choroid plexus epithelial cells (10).

**Perspectives and Significance**

Carnosine is a naturally-occurring dipeptide that has many physiological roles and the potential for pharmacological interventions (e.g., as a neuroprotective agent in ischemia-induced cerebral and renal injury). Carnosine is also a substrate for PEPT2, a transporter that has been shown to have significant effects on dipeptide/mimetic disposition, dynamics, and toxicity. The emergence of Pept2 null mice has provided a unique opportunity to evaluate the evolving role and relevance of PEPT2 as applied to carnosine disposition. This is the first study to report the effects of PEPT2 ablation on the endogenous levels of carnosine and the handling of exogenous carnosine under physiological in vivo conditions. Our findings are novel in demonstrating that, even though tissue and plasma levels of exogenous carnosine are reduced significantly in Pept2-deficient mice (and CSF levels increased), the body can adapt so that plasma and CSF concentrations of endogenous carnosine are maintained in the Pept2 null animals. The mechanisms for this adaptation are unclear at present but deserve further attention. This aspect, in addition to PEPT2 regulation under pathophysiological states, such as ischemia, will be addressed in subsequent studies.

**GRANTS**

This study was partially funded by National Institutes of Health Grants R01-GM-035498 (to D. E. Smith) and R01-NS-034709 (to R. F. Keep). M. A. Kamal was supported by the Pharmaceutical Sciences Training Program of the National Institutes of Health Grant T32-GM-007767. H. Jiang was supported by a fellowship from Zhejiang University and a scholarship from the China Scholarship Council.

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