British Journal of Nutrition (2006), **95**, 1221–1228
DOI: 10.1079/BJN20061793

Transepithelial flux of early and advanced glycation compounds across Caco-2 cell monolayers and their interaction with intestinal amino acid and peptide transport systems

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(Received 5 May 2005 – Revised 30 September 2005 – Accepted 10 November 2005)

Maillard products arise from condensation reactions between amino acids or proteins with reducing sugars during food processing. As ubiquitous components of human food, these early or advanced glycation products may be subject to intestinal absorption. The present study was performed to investigate the intestinal uptake of Maillard products and to determine whether they are substrates for peptide and amino acid transporters expressed at the apical membrane of Caco-2 cells. At a concentration of 10 mM, N^{ε}-(carboxymethyl)-L-lysine, N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-L-lysine, N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine and N^{ε}-(1-deoxy-D-fructosyl)-L-lysine inhibited the [^{14}C]glycylsarcosine uptake mediated by the H^+–peptide co-transporter PEPT1 by 13 to 45 %. For N^{ε}-(1-deoxy-D-fructosyl)-L-lysine, an inhibitory constant of 8·7 mM was determined, reflecting a low affinity to PEPT1 in comparison with natural dipeptides. Uptake of L-[^{3}H]lysine was weakly affected by N^{ε}-(carboxymethyl)-L-lysine, N^{α}-hippuryl-L-lysine and N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine but strongly inhibited by N^{ε}-(1-deoxy-D-fructosyl)-L-lysine (81 %). None of the Maillard products was able to inhibit the uptake of L-[^{3}H]leucine by more than 15 %. We also studied the transepithelial flux of Maillard products across Caco-2 cell monolayers cultured on permeable filters. The flux rates of Maillard products ranged from 0·01 to 0·3 %/cm^2 per h and were shown to be much lower than those of carrier substrates such as glycylsarcosine, L-proline and the space marker [^{14}C]mannitol. We conclude that the Maillard products investigated in the present study are neither transported by PEPT1 nor by carriers for neutral amino acids. The low transepithelial flux measured for these compounds most probably occurs by simple diffusion.

Advanced glycation reactions: Maillard products: Membrane transport: Resorption: Caco-2 cells

During heating or storage of food, the nutritional quality of proteins is significantly influenced by the so-called Maillard reaction (Friedman, 1996; Gerrard, 2002). This reaction, also referred to as non-enzymic browning or 'glycation', occurs between reducing carbohydrates or their degradation products with amino acid side chains. Primary targets of such post-translational modifications are the ε-amino group of lysine and the guanidino group of arginine (Fig. 1; Henle & Miyata, 2003).

Besides the 'early' glycation compounds, namely the amino-ketoses or 'Amadori compounds' N^{ε}-(1-deoxy-D-fructosyl)-L-lysine (FruLys) and N^{ε}-lactuloselysine, several reaction products of 'advanced' glycation reactions (advanced glycation end products; AGE) have been demonstrated in food (Henle, 2003). Quantification of individual glycation compounds might serve as a tool for controlling the impact of food processing and storage on the quality of foods (Krause *et al.* 2003).

It has been speculated that dietary Maillard compounds are a 'risk factor' for human health (Koschinsky *et al.* 1997; He *et al.* 1999). Hence, questions arise regarding the intake of dietary AGE via the daily food and their possible (patho)physiological role (Erbersdobler & Faist, 2003). The formation of AGE *in vivo* contributes to the pathogenesis of diabetes, uraemia and ageing (Raj *et al.* 2000; Henle & Miyata, 2003). However, since Maillard compounds are also produced endogenously in the human organism, their identification in human tissues and body fluids does not necessarily mean that these compounds had been taken up with the diet.

The daily intake of glycation compounds can be as high as 0·5 to 1·2 g for the Amadori compound FruLys and 25 to 75 mg for the AGE pyrraline and N^{ε}-(carboxymethyl)-L-lysine (CML) (Henle, 2003). Only preliminary studies concerning digestion, resorption and elimination of AGE-modified food proteins have been published; initial rat feeding studies using proteins radioactively labelled by heating with [^{14}C]glucose gave evidence for a very low resorption rate. Less than 3 % of the ingested radioactivity was measured in the urine of rats (Valle-Riestra & Barnes, 1970; Finot & Magnenat, 1981). Studies with human volunteers showed that the faecal excretion is about 1 % of orally administered FruLys and the

Abbreviations: AGE, advanced glycation end product; CML, N^{ε}-(carboxymethyl)-L-lysine; FruLys, N^{ε}-(1-deoxy-D-fructosyl)-L-lysine; Gly-Sar, glycylsarcosine; HipCML, N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine; HipFruLys, N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-L-lysine; HipLys, N^{α}-hippuryl-L-lysine; IC_{50}, 50 % inhibitory concentration.

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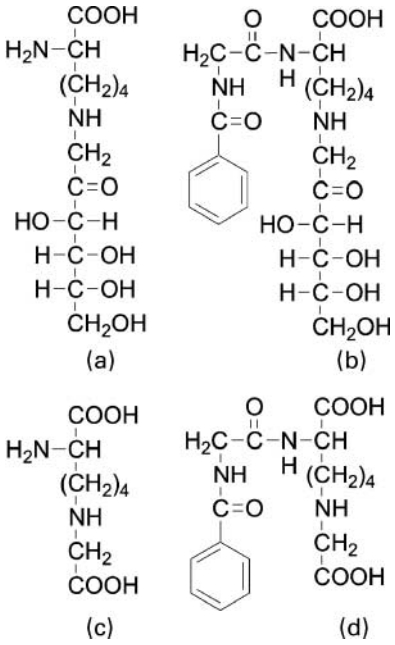

Fig. 1. Maillard compounds synthesised and used in the study. (a) N^{ε}-(1-deoxy-D-fructosyl)-L-lysine, (b) N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-L-lysine, (c) N^{ε}-(carboxymethyl)-L-lysine, (d) N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine.

urinary excretion about 3 % (Erbersdobler *et al.* 1991; Henle *et al.* 2000). The low faecal excretion is explained by limited proteolytic digestion and, more likely, metabolic activity of the intestinal flora (Erbersdobler *et al.* 1970; Wiame *et al.* 2002). In contrast to Amadori products, recent studies in our laboratory indicate enhanced bioavailability of selected AGE. For example, nearly all peptide-bound pyrraline supplied with the diet was found as free amino acid in the urine (Förster & Henle, 2003). Koschinsky *et al.* (1997) discussed that serum AGE levels, as measured with ELISA, can be influenced by a diet containing AGE.

For dietary Maillard products to elicit effects within the human body, absorption in effectual amounts across the intestinal epithelial barrier is required. To the best of our knowledge, studies on the transepithelial transport of individual glycation compounds have not yet been performed.

In the present study, we tested the hypothesis that Maillard products are potential substrates for amino acid or peptide transporters. It has been well established in recent years that at the apical membrane of enterocytes at least six different systems are responsible for the uptake of amino acids and di- and tripeptides originating from protein digestion. The peptide transporter PEPT1 expressed in the luminal membrane of enterocytes is driven by a transmembrane H^+ gradient and catalyses the co-transport of its substrates with H^+ (for a review, see Daniel, 2004). PEPT1's naturally occurring substrates are di- and tripeptides. The recognition of exogenous compounds such as β-lactam antibiotics by PEPT1 (Bretschneider *et al.* 1999) is based on the phenomenon that these compounds possess peptide-like chemical structures. PEPT1 accepts many amino acid derivatives and modified dipeptides (Börner *et al.* 1998; Abe *et al.* 1999; Brandsch *et al.* 2004; Neumann *et al.* 2004).

Similarly, amino acid transport systems such as B^0, $B^{0,+}$, $b^{0,+}$, y^+ and PAT1 are candidates for AGE transport because they also might accept modified amino acids (Hatanaka *et al.* 2004; Metzner *et al.* 2004).

To study the intestinal transport of Maillard products, quantitatively relevant Maillard products were synthesised and characterised spectroscopically. In competition assays *v.* radiolabelled reference substrates, their interaction with the carriers responsible for the uptake of L-lysine, L-leucine and dipeptides was determined. Moreover, we measured the total transepithelial net flux of several relevant Maillard products across Caco-2 cell monolayers.

Materials and methods

Materials

The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany). Cell culture media and supplements and trypsin solution were purchased from Life Technologies, Inc. (Karlsruhe, Germany). Fetal bovine serum was from Biochrom (Berlin, Germany). [Glycine-1-^{14}C]glycylsarcosine (Gly-Sar; specific radioactivity 1961 MBq/mmol) and L-[4,5-^{3}H]lysine (specific radioactivity 3404 GBq/mmol) were obtained from Amersham International (Little Chalfont, Bucks, UK), and L-[3,4,5^{3}H(N)]leucine (specific radioactivity 5549 GBq/mmol) from NEN™ Life Science Products, Inc. (Boston, MA, USA). N^{α}-hippuryl-L-lysine (HipLys), N^{α}-t-butyloxycarbonyl-L-lysine and N^{α}-acetyl-L-lysine were obtained from Bachem (Heidelberg, Germany). HPLC-grade methanol was from Riedel de Haen (Seelze, Germany). AG 50W-X8 ion exchange resin was from Bio-Rad Laboratories (Munich, Germany). HCl of 'ACS quality' was obtained from J. T. Baker (Deventer, The Netherlands). Furosine was from Neosystem (Strasbourg, France). Carboxypeptidase B (*EC* 3.4.17.2) (diisopropyl fluorophosphate-treated, 133 U/mg protein, 5 mg protein/ml) and all other chemicals were purchased from Fluka (Taufkirchen, Germany). The water used for the preparation of buffers and solutions was obtained with a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany). All other chemicals were of highest purity available.

Synthesis of Maillard products

N$^{\alpha}$-hippuryl-N$^{\varepsilon}$-(1-deoxy-D-fructosyl)-L-lysine. The method described by Krause *et al.* (2003) with the modifications according to Seifert *et al.* (2004) was used. Briefly, HipLys was brought to reaction with anhydrous glucose in methanol to N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-L-lysine (HipFruLys), followed by incubating the reaction mixture with carboxypeptidase B to remove remaining HipLys and subsequent purification of HipFruLys by semi-preparative reversed-phase-HPLC. Purity was checked using liquid chromatography–MS.

N$^{\varepsilon}$-(1-deoxy-D-fructosyl)-L-lysine. The protocol published by Krause *et al.* (2003) was applied. Briefly, a mixture of N$^{\alpha}$-Boc-L-lysine and glucose was incubated in the dry state, followed by semi-preparative cation-exchange chromatography using a mixture of pyridine and acetic acid as eluent. Liquid chromatography–MS and NMR were performed as described later.

N^{α}-hippuryl-N^{ε}-L-(carboxymethyl)-L-lysine. The synthesis was performed according to Liardon *et al.* (1987) with isolation of the product according to Krause *et al.* (2003) and Seifert *et al.* (2004). Dissolved in 20 ml water were 5 mmol HipLys and 6·5 mmol glyoxylic acid monohydrate and the pH value was adjusted to 8·7 with 1 M-NaOH. After adding 52 mg palladium on charcoal, the reaction mixture was incubated for 29 h at 23°C and 20 bar under continuous stirring in a high-pressure autoclave. After this, the pH was adjusted to 7·0 using 2 M-HCl and the solvent was removed under reduced pressure. The dried residue was dissolved in 20 ml water and applied to a column (180 × 18 mm), filled with anion-exchanger Dowex 1X8 (acetate form; Bio-Rad, Munich, Germany), pre-equilibrated with 2 litres 1 M-NaOH, 1 litre 1 M-acetic acid, 2 litres 2 M-acetic acid and 1 litre water at a flow rate of 60 ml/h. After sample injection, the column was eluted with 350 ml 0·5 M-acetic acid, 1 litre 1 M-acetic acid and finally with 700 ml 1·5 M-acetic acid at a flow rate of 30 ml/h. Fractions of 6 ml were collected. The presence of N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine (HipCML) in the collected fractions was detected by TLC with UV detection and reversed-phase HPLC as described earlier. Fractions containing HipCML were pooled, evaporated under reduced pressure, lyophilised and stored at −20°C.

N^{ε}-(carboxymethyl)-L-lysine. The synthesis was performed as described earlier for HipCML, but using N^{α}-acetyl-L-lysine instead of HipLys. After incubation in a high-pressure autoclave, the catalyst was removed by filtration, and after adding 20 ml 6 M-HCl, the solution was hydrolysed for 2 h at 120°C. After cooling, the solution was evaporated to dryness under reduced pressure. The residue was dissolved in 50 ml water, membrane filtered (45 μm) and applied to a column (180 × 18 mm) filled with cation-exchanger AG 50WX8 (H^+ form; Bio-Rad, Munich, Germany), pre-equilibrated with 250 ml 6 M-HCl and 500 ml water. At a flow rate of 30 ml/h, the column was washed with 380 ml 0·01 M-HCl to remove unbound material, followed by elution with 650 ml 1 M-HCl, 550 ml 1·5 M-HCl and 700 ml 2 M-HCl. Fractions of 6 ml were collected. The presence of CML in the collected fractions was detected by TLC with ninhydrin as described later. Fractions containing CML were pooled, evaporated under reduced pressure, lyophilised and stored at −20°C.

Cell culture

Caco-2 cells were routinely cultured in 75 cm^2 culture flasks with Minimum Essential Medium supplemented with 10 % fetal bovine serum, gentamicin (45 μg/ml) and 1 % non-essential amino acid solution (Börner *et al.* 1998; Knütter *et al.* 2004; Metzner *et al.* 2004). Subconfluent cultures (90 % of confluence) were treated for 5 min with Dulbecco's PBS followed by a 2 min incubation with trypsin solution. For most experiments, the cells were seeded in 35 mm disposable petri dishes (BD Biosciences, Heidelberg, Germany) at a density of $0{\cdot}8 \times 10^6$ cells per dish. The uptake measurements were performed on day 7 after seeding. Protein content per dish was determined according to the Bradford method.

Caco-2 cells were also cultured on permeable polycarbonate Transwell® cell culture inserts (diameter 24·5 mm, pore size 3 μm; Costar GmbH, Bodenheim, Germany) (Bretschneider *et al.* 1999; Metzner *et al.* 2004). Subcultures were started at a cell density of 43 000 cells/cm^2 and cultured for 21 d. The lower (receiver) compartment contained 2·6 ml medium and the upper (donor) compartment 1·5 ml medium. The transepithelial electrical resistance was measured at day 21 using a Millicell ERS (Millipore Intertech, Bedford, MA, USA).

Transport studies

Uptake of [^{14}C]Gly-Sar, L-[^{3}H]leucine and L-[^{3}H]lysine in Caco-2 cells cultured on plastic dishes was measured at room temperature as described earlier (Börner *et al.* 1998; Bretschneider *et al.* 1999; Knütter *et al.* 2004). The uptake buffer contained 25 mM 2(*N*-morpholino) ethane sulfonic acid–tri(hydroxymethyl)-aminomethane (pH 6·0), 140 mM-NaCl, 5·4 mM-KCl, 1·8 mM-$CaCl_2$, 0·8 mM-$MgSO_4$, 5 mM-glucose, radiolabelled reference substrates and unlabelled compounds at a concentration of mostly 10 mM. After incubation for 10 min, the cells were quickly washed four times, dissolved in Igepal CA-630 buffer and prepared for liquid scintillation spectrometry. The non-saturable component of [^{14}C]Gly-Sar uptake (diffusion, adherent radioactivity) determined by measuring the uptake of [^{14}C]Gly-Sar in the presence of 30 mM-unlabelled Gly-Sar represented 11 % of the total uptake. This value was taken into account during non-linear regression analysis of inhibition constants.

Transepithelial flux of Maillard products was measured as follows (Bretschneider *et al.* 1999; Metzner *et al.* 2004). All experiments were performed at day 21 after seeding at 37°C in a shaking water-bath. After washing the inserts with buffer (25 mM-HEPES–tri(hydroxymethyl)-aminomethane (pH 7·5), 140 mM-NaCl, 5·4 mM-KCl, 1·8 mM-$CaCl_2$, 0·8 mM-$MgSO_4$, 5 mM-glucose) for 10 min, uptake was started by adding uptake buffer (pH 6·0; 1·5 ml) containing compounds (mostly 5 mM) to the donor side. At time intervals of 10, 30, 60 and 120 min, 200 μl samples were taken from the receiver compartment and replaced with fresh buffer (pH 7·5). Samples were stored until analysis. After 2 h, the filters were quickly washed four times with ice-cold uptake buffer, cut out of the plastic insert and stored in 1 ml water and frozen.

L-Lysine, CML and FruLys were quantified in the uptake buffer and in the samples taken from the receiver compartment using ion-exchange chromatography and ninhydrin detection. HipFruLys and HipCML were measured using reversed-phase HPLC with direct UV detection (see later). Both methods allowed selective and sensitive measurement of the analytes within the samples.

The integrity of the Caco-2 cell monolayers grown on permeable filters in the presence of Maillard products was verified by measuring the transepithelial electrical resistance. This method allows us to exclude membrane-damaging effects of the compounds which would lead to false high flux rates.

Thin-layer chromatography

TLC was carried out on pre-coated TLC plates SIL G-25 (Machery-Nagel, Düren, Germany). The solvent system was acetonitrile–water–acetic acid (80:20:20, by vol.). Visualisation was achieved by spraying the plates with 0·1 % ninhydrin in ethanol, followed by heating at 90°C for 5 min or by

spraying with 1 % 2,3,5-triphenyl-2H-tetrazolium chloride in 1·0 M-NaOH, followed by heating at 50°C for 2 min. Alternatively, UV-absorbing compounds were detected under a UV lamp.

Amino acid analysis

Analysis of furosine, CML and the common amino acids was performed with an Alpha Plus amino acid analyser (LKB Biochrom, Cambridge, UK) using a 125 × 4·6 mm peek column filled with ion-exchange resin series 686 (Laborbedarf K. Grüning, Olching, Germany). The composition of buffers, ninhydrin reagent and the running conditions are described by Henle *et al.* (1991). For external calibration of CML, N^{ε}-(carboxymethyl)-L-hippuryllysine was used as standard after acid hydrolysis and with glycine as internal reference.

High-pressure liquid chromatography

HPLC was performed with a gradient pump system (Knauer, Berlin, Germany) with online degasser, K1500 solvent organiser, K1001 pump, dynamic mixing chamber and a K2501 Knauer variable wavelength detector. Analytical separation of hippuryl derivatives was achieved using a stainless-steel column, 250 × 4·6 mm, filled with Knauer Eurospher 100, RP18-material of 5 μm particle size, with integrated guard column (5 × 4 mm) filled with the same material (Knauer, Berlin, Germany). The flow rate was 1·0 ml/min at 20°C; detection was performed at 230 nm. Solvent A was 0·01 M-sodium phosphate buffer (pH 7·0); solvent B was methanol. A linear gradient from 2 % B to 6 % B in 25 min was used.

Semi-preparative separation was performed using a stainless-steel column (250 × 8 mm) with a guard column (30 × 8 mm), both filled with Knauer Eurospher 100 RP18-material of 10 μm particle size (Knauer, Berlin, Germany). Flow rate was 1·5 ml/min at 20°C; detection was performed at 280 nm. The first chromatographic stage was performed isocratically with a mixture of 0·01 M-sodium phosphate buffer (pH 7·0) and methanol (90:10, v/v). The second stage was performed isocratically with a mixture of 0·05 M-acetic acid and methanol (90:10, v/v).

Nuclear magnetic resonance spectroscopy, mass spectrometry and elemental analysis

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX 500 instrument (Bruker, Rheinstetten, Germany). Proton chemical shifts are relative to internal dimethyl sulfoxide for DMSO-d6 solutions or to internal deuterium-labelled water for ^{2}H-labelled water solutions. Carbon chemical shifts are given relative to DMSO-d6 or to external standard tetramethylsilane for ^{2}H-labelled water solutions. Assignments of ^{1}H and ^{13}C signals were based on two-dimensional NMR experiments as described in Krause *et al.* (2003).

For MS, a coupled liquid chromatography-electrospray ionisation MS system (Esquire-LC; Hewlett Packard-Bruker, Bremen, Waldbronn, Germany) was used; for elemental analysis a Euro EA 3000 elemental analyser (Eurovector, Milan, Italy) was used.

Data analysis

Experiments were done in duplicate or triplicate and each experiment was repeated two to three times. Results are given as means and their standard errors of the mean. Values of 50 % inhibitory concentration (IC_{50} values; i.e. concentration of unlabelled compounds necessary to inhibit 50 % of [^{14}C]Gly-Sar carrier-mediated uptake) were determined by non-linear regression (Knütter *et al.* 2004). Flux data were calculated after correction for the amount taken out by linear regression of appearance in the receiver well *v*. time.

Results

Syntheses

Syntheses resulted in all cases in chromatographically pure compounds. Results of NMR and MS were in agreement with published data (Krause *et al.* 2003) and confirmed the synthesis of these compounds. Elemental analysis gave the following results:

HipFruLys: $C_{21}H_{31}N_3O_9 \times 0{\cdot}60\ CH_3COOH \times 1{\cdot}30\ H_2O$ (molecular weight 528·94 Da); calculated: C, 50·41 %; H, 6·86 %; N, 7·94 %; found: C, 50·43 %; H, 6·54 %; N, 7·93 %;

FruLys: $C_{12}H_{24}N_2O_7 \times 0{\cdot}80\ CH_3COOH \times 0{\cdot}80\ CH_3OH \times 0{\cdot}10\ C_2H_5COCH_3$ (molecular weight 389·21 Da); calculated: C, 45·67 %; H, 8·08 %, N, 7·20 %; found: C, 45·40 %; H, 7·99 %; N, 7·17 %;

HipCML: $C_{17}H_{23}N_3O_6 \times 0{\cdot}40\ CH_3COOH \times 0{\cdot}90\ H_2O$ (molecular weight 405·62 Da); calculated: C, 52·71 %; H, 6·56 %; N, 10·36 %; found: C, 52·78 %; H, 6·38 %; N, 10·41 %;

CML: $C_8H_{16}N_2O_4 \times 0{\cdot}30\ 0{\cdot}8\ H_2O \times 0{\cdot}20\ NH_4Cl \times 2\ HCl$ (molecular weight 293·25 Da); calculated: C, 32·77 %; H, 6·67 %; N, 10·51 %; found: C, 32·90 %; H, 6·81 %; N, 10·49 %.

All isolates contained small amounts of water and acetate, the latter resulting from chromatographic separation of the final products. FruLys additionally contained traces of methanol. Free CML was purified via ion-exchange chromatography as the corresponding dihydrochloride, containing traces of ammonia.

Inhibition of [^{14}C]glycylsarcosine uptake

We investigated whether Maillard products represent potential substrates for the intestinal H^+–peptide co-transporter PEPT1. This was done in competition experiments using radiolabelled Gly-Sar as reference substrate of the transport system. Several Maillard products at a concentration of 10 mM were able to inhibit H^+-dependent uptake of [^{14}C]Gly-Sar. CML, HipFruLys, HipCML and FruLys inhibited [^{14}C]Gly-Sar uptake into Caco-2 cells by 13 to 45 % (Fig. 2). HipLys, which was used as control for unmodified peptide-bound lysine, behaved similarly. Lysinoalanine had no significant effect. The inhibitor showing the highest apparent inhibitory potency was FruLys. In a dose–response type competition assay we determined an IC_{50} value of 8·7 (SEM 2·2) mM for [^{14}C]Gly-Sar uptake inhibition (Fig. 3). This value reflects a rather low affinity of FruLys to PEPT1 according to our classification (Brandsch *et al.* 2004). For comparison, we also measured the interaction of L-lysine and the dipeptide Ala-Lys with PEPT1. Whereas the amino acid L-lysine shows no affinity

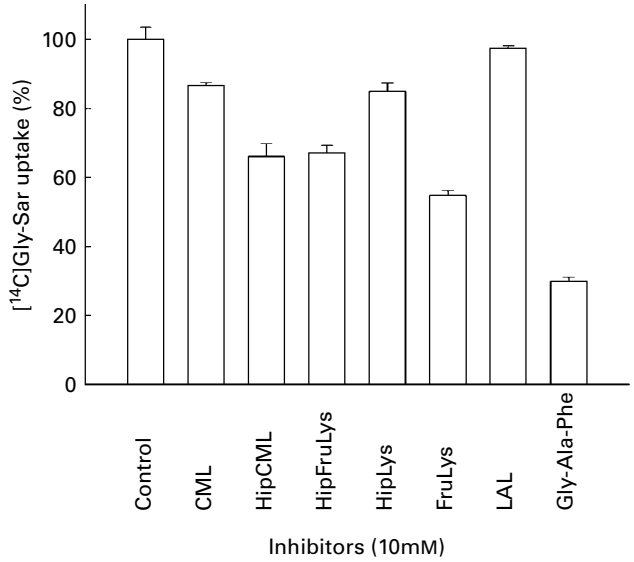

Fig. 2. Inhibition of [^{14}C]glycylsarcosine ([^{14}C]Gly-Sar) uptake by Maillard products and Gly-Ala-Phe in Caco-2 cells. Uptake of 10 μM-[^{14}C]Gly-Sar was measured for 10 min in monolayer cultures of Caco-2 cells at pH 6·0 in the absence (control) or presence of 10 mM of the compounds. Uptake of [^{14}C]Gly-Sar measured in the absence of the inhibitors was taken as 100 % (165 (SEM 8) pmol/10 min per mg protein). Data are means (n 3–4), with their standard errors represented by vertical bars. CML, N^{ε}-(carboxymethyl)-L-lysine; HipCML, N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine; HipFruLys, N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-L-lysine; HipLys, N^{α}-hippuryl-L-lysine; FruLys, N^{ε}-(1-deoxy-D-fructosyl)-L-lysine; LAL, lysinoalanine.

to PEPT1, Ala-Lys represents a high affinity PEPT1 substrate (IC_{50} 0·31 (SEM 0·07) mM).

Inhibition of amino acid uptake

In a similar approach, we tested the ability of the Maillard products to inhibit L-leucine and L-lysine uptake. Uptake of L-[^{3}H]lysine was weakly inhibited by CML (30 mM) and HipLys (10 mM). FruLys, unlabelled L-lysine and L-leucine inhibited the uptake of L-[^{3}H]lysine by 81, 90 and 84 %, respectively. HipCML had no effect (Fig. 4).

None of the compounds was able to inhibit the uptake of L-[^{3}H]leucine by more than 15 % (Fig. 5). For comparison and to verify the experimental conditions, inhibition of L-[^{3}H]leucine uptake by L-leucine and L-lysine was measured. Uptake was inhibited by L-leucine (10 mM) by 81 % and by L-lysine (10 mM) by 18 %.

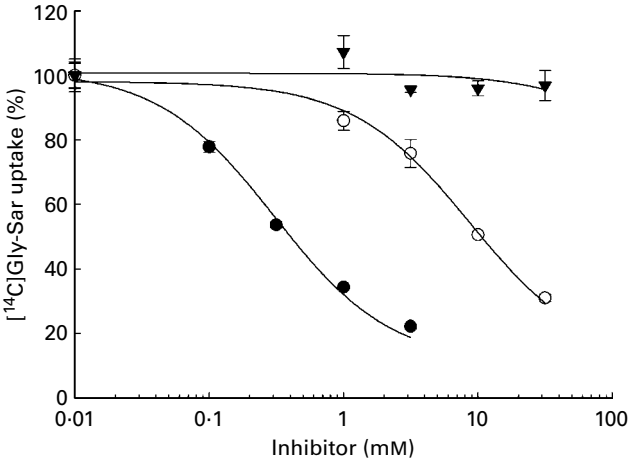

Fig. 3. Inhibition of [^{14}C]glycylsarcosine ([^{14}C]Gly-Sar) uptake in Caco-2 cells. Uptake of 10 μM-[^{14}C]Gly-Sar was measured for 10 min in monolayer cultures of Caco-2 cells at pH 6·0 in the absence (control) or presence of 0–31·6 mM-Ala-Lys (●), L-lysine (▼) and N^{ε}-(1-deoxy-D-fructosyl)-L-lysine (○). Data are means (n 3–4), with their standard errors represented by vertical bars.

Transepithelial flux

At a concentration of 5 mM, none of the compounds showed negative effects on the transepithelial electrical resistance of Caco-2 monolayers (Table 1). With regard to their own flux, even after 2 h the basolateral amounts of CML and HipFruLys were below the detection limit (Table 1). No flux rates could be calculated. Flux rates of HipCML, FruLys and lysinoalanine were between 0·008 and 0·26 %/cm^2 per h (Table 1). Hence, only the flux of HipCML was higher than the flux of the space marker [^{14}C]mannitol.

No significant amounts of Maillard products were found in Caco-2 cell monolayers cut out of the inserts after 2 h. The results were supported by quantification of the Maillard products in the donor compartment during the experiments. No decrease of the starting concentration of 5 mM due to transport into the lower compartment could be observed. These measurements concomitantly confirmed the stability of the compounds over 2 h in the uptake buffer contacting the brush-border membrane of the cells.

Discussion

The present results show that the intestinal transport of the Maillard products and AGE investigated in the present study

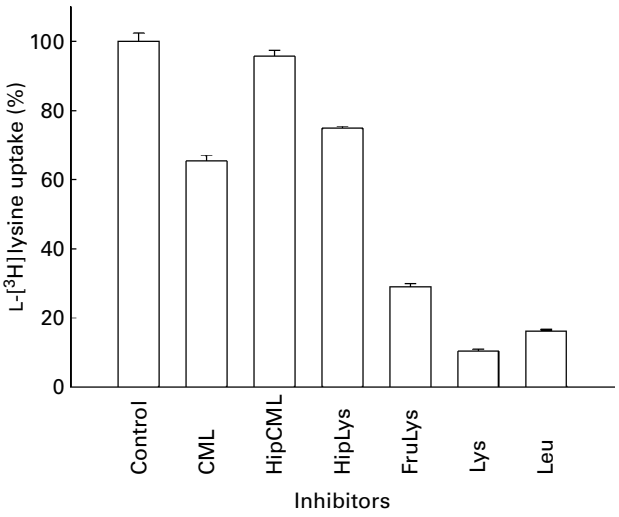

Fig. 4. Inhibition of L-[^{3}H]lysine uptake by Maillard products, and L-leucine (Leu) and L-lysine (Lys) in Caco-2 cells. Uptake of 2 nM-L-[^{3}H]lysine was measured for 10 min in monolayer cultures of Caco-2 cells at pH 6·0 in the absence (control) or presence of 10 mM of the compounds (N^{ε}-(carboxymethyl)-L-lysine (CML), 30 mM). Uptake of L-[^{3}H]lysine measured in the absence of the inhibitors (49 (SEM 7) fmol/10 min per mg protein) was taken as 100 %. Data are means (n 3–4), with their standard errors represented by vertical bars. HipCML, N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine; HipLys, N^{α}-hippuryl-L-lysine; FruLys, N^{ε}-(1-deoxy-D-fructosyl)-L-lysine.

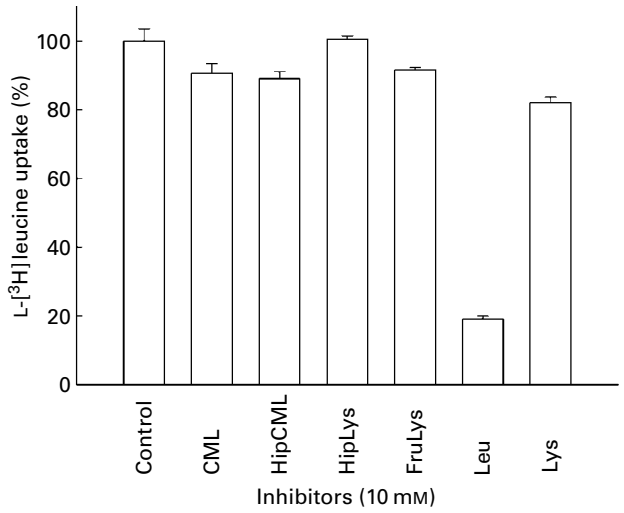

Fig. 5. Inhibition of L-[^{3}H]leucine uptake by Maillard products and L-leucine (Leu) and L-lysine (Lys) in Caco-2 cells. Uptake of 1·2 nM-L-[^{3}H]leucine was measured for 10 min in monolayer cultures of Caco-2 cells at pH 6·0 in the absence (control) or presence of 10 mM of the compounds. Uptake of L-[^{3}H]leucine measured in the absence of the inhibitors (120 (SEM 6) fmol/10 min per mg protein) was taken as 100 %. Data are means (n 4), with their standard errors represented by vertical bars. CML, N^{ε}-(carboxymethyl)-L-lysine; HipCML, N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine; HipLys, N^{α}-hippuryl-L-lysine; FruLys, N^{ε}-(1-deoxy-D-fructosyl)-L-lysine.

Table 1. Transepithelial flux of Maillard products (all 5 mM) and reference compounds (10 nM–30 μM) across Caco-2 cell monolayers*

(Mean values with their standard errors)

	Transepithelial resistance ($\Omega \times cm^2$)		Transepithelial flux (%/cm^2 per h)	
Compound	Mean	SEM	Mean	SEM
Control	605	8		
HipCML	592	42	0·26	0·08
CML	610	18	<dl	
HipFruLys	645	22	<dl	
FruLys	679	9	0·008	0·003
LAL	707	11	0·02	0·01
[^{14}C]mannitol			0·07	0·002†
[^{14}C]Gly-Sar			1·4	0·1†
l-[^{3}H]proline			5·3	0·4‡

HipCML, N^{α}-hippuryl-N^{ε}-(carboxymethyl)-L-lysine; CML, N^{ε}-(carboxymethyl)-L-lysine; <dl, below detection limit (HPLC, amino acid analysis); HipFruLys, N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-L-lysine; FruLys, N^{ε}-(1-deoxy-D-fructosyl)-L-lysine; LAL, lysinoalanine; Gly-Sar, glycylsarcosine.

* Substances were added to the donor compartment (1·5 ml) of Transwell systems in uptake buffer (pH 6·0). Samples (200 μl) were taken from the receiver compartment (pH 7·5) and analysed as described (p. 1224) or by liquid scintillation counting (n 4; resistance, n 3).

† Value from Bretschneider *et al.* (1999).

‡ Value from Metzner *et al.* (2004).

is low and not mediated by amino acid and peptide carriers. The type of compounds and the concentrations applied here *in vitro* correspond to the human physiological situation *in vivo* after food intake. We selected the quantitatively predominant Amadori product of lysine FruLys, its oxidative degradation product CML and, to investigate the influence of *N*-terminal modification, their corresponding hippuryl derivatives HipFruLys and HipCML. With regard to the concentrations, the range of 1 to 30 mM is conceivable considering the fact that, for example, the daily CML intake in human consumers can be as high as 30 mg. Similarly, Amadori products of L-lysine in milk can reach concentrations as high as 2 mM (Henle, 2003).

CML, HipCML, HipFruLys, HipLys and lysinoalanine inhibit the total [^{14}C]Gly-Sar uptake in Caco-2 cells only by 3 to 34 %. Since it has been shown unequivocally that the uptake of [^{14}C]Gly-Sar into Caco-2 cells is mediated by a single transport system which has been identified as the low-affinity, high-capacity intestinal system PEPT1 (Daniel, 2004; Brandsch *et al.* 2004) we conclude that PEPT1 can be ruled out as transport system for the Maillard products.

The affinity constant of FruLys, the strongest inhibitor, at PEPT1 was 9 mM. It should be noted that in this assay natural di- and tripeptides display IC_{50} values of 0·1 to 0·3 mM as shown here for Ala-Lys. Inhibitors with IC_{50} values of >15 mM are not considered substrates; for β-lactam antibiotics it has been shown that in general only those compounds with an IC_{50} value at PEPT1 of <14 mM are transported by this carrier across the Caco-2 cell monolayer and that the transport rate corresponds very well with the oral availability. Compounds with an IC_{50} (approximately K_i in these assays) of >15 mM have to be administered parenterally. Hence, FruLys can be considered a low-affinity PEPT1 inhibitor or a low-affinity PEPT1 substrate.

With the possible exception of FruLys, the Maillard products tested in the present study are not substrates for the amino acid carriers responsible for the uptake of the amino acids L-lysine and L-leucine. None of the compounds was able to inhibit the uptake of L-[^{3}H]leucine by more than 15 %. Uptake of L-[^{3}H]lysine was not inhibited by HipCML; low inhibition was observed with CML (30 mM) and HipLys (10 mM). FruLys (10 mM), on the other hand, inhibited L-lysine uptake strongly by 81 %.

With regard to the amino transport systems involved in L-leucine and L-lysine uptake (Brandsch & Brandsch, 2003) we conclude from the substrate specificity results that L-lysine is mainly transported by system $B^{0,+}$or system $b^{0,+}$ because L-leucine inhibited the L-lysine uptake strongly by 84 %. L-Leucine seems to be transported mainly by B^0 because the L-[^{3}H]leucine uptake was inhibited by 81 % by unlabelled L-leucine itself but only 18 % by 10 mM-L-lysine.

Measurement of Gly-Sar, L-lysine and L-leucine uptake in the presence of Maillard products only allows estimation of their potency for dipeptide or amino acid uptake inhibition, for example, their apparent affinity to the systems. The results do not allow the conclusion that the effective compound inhibiting uptake of reference substrates are indeed transported by the systems across the cell membrane. We therefore performed the flux studies described. Importantly, the Maillard products tested in the present study did not affect the integrity of the Caco-2 monolayers. They did not damage the cells or the cell junctions. In addition, they do not seem to affect the cell membrane fluidity to a significant extent. It is therefore concluded from the results that the transepithelial electrical resistance was not changed by the compounds.

The apical to basolateral transepithelial flux rates of Maillard products at apical concentrations of 5 mM ranged from 0·01 to 0·3 %/cm^2 per h. They were much lower than the flux rates of Gly-Sar, L-phenylalanine (data not shown), L-proline

and mannitol: In previous studies we determined a reference flux rate of [^{14}C]mannitol across Caco-2 cell monolayers of 0·07 (SEM 0·002) %/cm^2 per h (Bretschneider *et al.* 1999). For comparison, the flux of [^{14}C]Gly-Sar was found to be 20-fold higher: At a substrate concentration of 30 μM, 616·9 (SEM 39·6) pmol/cm^2 per h was measured at the receiver side of the monolayers (1·4 %/cm^2 per h). Flux of L-[^{3}H]proline in another study was 5·3 (SEM 0·4) %/cm^2 per h (Metzner *et al.* 2004). Mannitol serves in such studies as a non-transported space marker. For Gly-Sar and L-proline, active, proton-dependent uptake at the apical membrane has been demonstrated unequivocally (Daniel, 2004; Knütter *et al.* 2004; Metzner *et al.* 2004). Comparing these flux rates, we have to conclude that the Maillard products tested do not reach the basolateral side of enterocytes in significant amounts.

Even though FruLys displays a relatively high affinity to the L-lysine transport system(s), its transport across the epithelium is negligible. FruLys possesses a free α-amino terminus and carboxy terminus. As we have shown recently, side-chain-protected Lys-Ala dipeptides such as Lys(Boc)-Ala and Lys(z)-Ala also display affinity to PEPT1 but are not transported by the system (Knütter *et al.* 2004). Abe *et al.* (1999) synthesised and tested dipeptide analogues conjugated at the ε-amino group of L-lysine in Val-Lys or Lys-Sar with fluorescent compounds such as fluorescein isothiocyanate and coumarin-3-carboxylic acid. These analogues inhibited the Gly-Sar uptake by Caco-2 cells with very high affinity but are not transported. In further studies, we will test whether FruLys as the C-terminal group of a dipeptide is recognised and transported by PEPT1; Kottra *et al.* (2002) have shown that the binding site of PEPT1 is asymmetric in the sense that side-chain modifications of L-lysine are tolerated when L-lysine is the C-terminal amino acid.

In conclusion, the present study shows that the transepithelial net flux of Maillard products across Caco-2 cell monolayers is low. Carriers for dipeptides, L-lysine and L-leucine do not seem to be involved. The integrity of cell monolayers was not affected by Maillard products at a concentration of 5 mM. The low transepithelial flux measured for these compounds occurs most probably by simple diffusion.

Acknowledgements

The present study was supported by Land Sachsen-Anhalt grant 3505A/0403L and by Bundesministerium für Bildung und Forschung grant no. 0312750A.

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