Uptake of *S*-(3-Amino-3-oxopropyl)-cysteine by Caco-2 Cells

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Acrylamide is a reactive neurotoxin with a high intestinal bioavailability. Recently we have shown that under the pH regime of the gut acrylamide can react with proteins and that this reaction reduces the uptake of acrylamide in a gut model. On the other hand, using radioactive labeled acrylamide, Bjellaas *et al.* [Toxicol. Sci. **100**, 374–380 (2007)] showed that *in vivo* the vast majority of orally administered acrylamide is absorbed and excreted as *N*-acetyl-*S*-(3-amino-3-oxopropyl)-cysteine with the urine. Therefore, we tested whether intestinal proteases can degrade a protein with acrylamide bound to cysteine residues. Furthermore we tested whether the product of this reaction, *S*-(3-amino-3-oxopropyl)-cysteine, can pass the intestinal barrier. Here we showed that *S*-(3-amino-3-oxopropyl)-cysteine is indeed a product of proteolytic degradation of acrylamide-treated proteins. Using Caco-2 cells as a gut model, we further showed that the non-protein amino acid *S*-(3-amino-3-oxopropyl)-cysteine is a substrate for the neutral and cationic amino acid transporter system. Hence we concluded that protein-bound acrylamide can be released in the intestine and that the resulting product *S*-(3-amino-3-oxopropyl)-cysteine is transported through the intestinal barrier and later excreted via the urine.

Key words: Acrylamide, Intestinal Uptake, Amino Acid Transport System

Introduction

Acrylamide is a neurotoxin and presumably cancer-causing agent that can be released during thermal food processing (Mottram *et al.*, 2002; Stadler *et al.*, 2002). Orally administered acrylamide is highly bioavailable (Doerge *et al.*, 2007; Tareke *et al.*, 2006) and passes through monolayers of intestinal Caco-2 cells via passive diffusion (Schabacker *et al.*, 2004; Zodl *et al.*, 2007). As an α,β-unsaturated, electrophilic molecule acrylamide can bind to DNA and proteins under alkaline conditions. Covalent binding to proteins was also suggested to be the molecular mechanism of acrylamide neurotoxicity (LoPachin and Barber, 2006). Like many other electrophilic toxins acrylamide is detoxified via the cytochrome P450 2E1 pathway and via conjugation to glutathione. Glutathione-bound acrylamide is further metabolized to *S*-(3-amino-3-oxopropyl)-cysteine [cysteine-acrylamide (CA)] and *N*-acetyl-*S*-(3-amino-3-oxopropyl)-cysteine (NAPC). NAPC eventually is excreted with the urine.

Abbreviations: CA, *S*-(3-amino-3-oxopropyl)-cysteine; GST, glutathione-S-transferase; ESI-MS, electrospray ionization-mass spectrometry; NAPC, *N*-acetyl-*S*-(3-amino-3-oxopropyl)-cysteine; RT, room temperature.

Much less is known about the fate of acrylamide in the intestine. Recently, we have shown that acrylamide also reacts with common food proteins under the pH conditions of the gut (Schabacker *et al.*, 2004). Moreover, we have demonstrated that pre-incubation of acrylamide with proteins reduces the uptake of acrylamide in Caco-2 cell monolayers. These data indicate that proteins in the diet may reduce the concentration of acrylamide in the gut and consequently reduce the uptake of acrylamide. Recently, Bjellaas *et al.* (2007), however, reported that 80% of the acrylamide that was fed to mice and 92% of the acrylamide that was injected subcutaneously was excreted as acrylamide metabolites with the urine. These data would indicate that administration of acrylamide with the food actually reduces the absorption of acrylamide only to a low extent.

In the present study we test the hypothesis that protein digestion in the intestine liberates protein-bound acrylamide in the form of CA. CA is a non-protein amino acid and it is likely that it is a substrate for an amino acid transporter system with broad substrate specificity. Therefore, CA might be transported through the intestinal barrier and later excreted with the urine. Using a set of intes-

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tinal proteases we show that digestion of proteins with bound acrylamide results in release of CA *in vitro* and that synthetic CA in turn is transported through Caco-2 cells via the neutral and cationic amino acid transporter systems.

Material and Methods

Synthesis of CA

0.5 g synthesis grade cysteine and 2.5 g acrylamide were dissolved in 30 ml 60 mM $NaHCO_3$, pH 8.3, and 70 ml ethanol. The reaction mixture yielding CA was incubated over night at room temperature (RT). CA was collected by centrifugation at $6000 \times g$ for 10 min, washed with 70% ethanol and further purified from remaining cysteine by preparative silica gel TLC using H_2O/butanol/acetic acid (50:40:10) as mobile phase [R_f (CA) = 0.16, R_f (cysteine) = 0.5]. Correct synthesis of CA was confirmed by ESI-MS. The product was dried under a steam of N_2 and kept at −70 °C in the dark until use. ^{35}S-CA was synthesized in a similar manner using ^{35}S-cysteine as starting material.

Alkylation and digestion of soybean trypsin inhibitor (SBTI)

SBTI (Sigma-Aldrich GmbH, Schnelldorf, Germany) was chosen because it is a small protein with a high number of cysteine residues and because it is a protein, which naturally occurs in the human diet. 50 mg SBTI were dissolved in reaction buffer [100 mM tris(hydroxymethyl)aminomethane-HCl, pH 8.8, 2% SDS, 0.07% 2-mercaptoethanol] and incubated at 95 °C for 5 min. 2% (w/v) acrylamide was added and the reaction mixture was incubated at 37 °C for 1 h. The alkylated protein was precipitated with acetone and intensively washed with 70% 1-propanol. The protein pellet was dried under a steam of N_2 in order to remove remaining 1-propanol.

Alkylated SBTI was resuspended in 10 mM HCl (pH 2). Pepsin (Merck, Darmstadt, Germany) was added to a final content of 1% (w/v) and the digestion was incubated over night at 37 °C. Then, the pH value was adjusted to 8 with solid $(NH_4)_2CO_3$, pancreatin, elastase, and chymotrypsin were added [1% (w/v) each] and the digestion was incubated over night at 37 °C. Carboxypeptidase A was added and the digestion was incubated at 4 °C for 6 h. The digestion of the protein was monitored by SDS-PAGE and TLC using dansylchloride as colour reagent. Released amino acids were precipitated with acetone and dissolved in 50% acetonitrile, 2% formic acid and analyzed by ESI-MS.

Mass spectrometry

Amino acids and acrylamide were analyzed using a VG Quattro II electrospray ionization tandem mass spectrometer (Micromass UK Ltd, Cheshire, UK). The ESI-mass spectrometer was operated in the positive electrospray mode. The dry gas (N_2) flow rate was set to 350 l/h and the spray gas to 13 l/h. Capillary voltage and temperature were set to 3.5 kV and 80 °C, respectively. For bombardment, argon 4.8 (Linde Gas, München, Germany) at a pressure of 0.2 bar was used. Samples were dissolved in 50% acetonitrile and 2% formic acid and applied with an A99 syringe pump (Razel, Georgia, VT, USA) at a flow rate of 35 μl/min. Calibration of the mass spectrometer with purified CA showed that the detection limit of CA in our system is 3 ng/μl (S/N = 3).

Cell culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 20% fetal bovine serum, 1 mM sodium pyruvate, 10 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biowhittaker, Walkersville, MD, USA). Cells were cultured in 75 cm^2 flasks (Greiner bio-one, Frickenhausen, Germany) and incubated at 37 °C with 5% CO_2 and 90% relative humidity. The culture medium was changed every second day. The cell viability was tested with standard MTT and neutral red assays.

Uptake and permeability assay

Cell monolayers were used between 14–21 and 21–28 d post-seeding for uptake and transport studies, respectively. Cells were harvested with 0.25% trypsin-EDTA and seeded at a density of 60,000 cells/cm^2 in 24-well plates (2.0 cm^2 surface area; Greiner bio-one) for uptake studies, or on Transwell polyester membrane filters (0.4 μm pore size, 1.0 cm^2 surface area, Costar 3460; Fisher Scientific GmbH, Frickenhausen, Germany) for transport studies. Both assays were conducted in Krebs-Ringer buffer containing 10 mM HEPES, pH 7.4, 142 mM NaCl, 3 mM KCl, 1.8 mM K_2HPO_4, 1.3 mM $MgCl_2$, 1.4 mM $CaCl_2$ and 4 mM D(+)-glucose. For all uptake studies cells were washed

twice with Krebs-Ringer buffer and then incubated for 15 min. The uptake of ^{3}H-labeled amino acids (5–10 nM) was tested after 25 or 45 min in the presence and absence of 1 mM CA. The uptake was arrested after aspirating the uptake buffer and immediately washing the cells twice with ice-cold buffer lacking substrate. For comparison, a molar surplus of 10^6 times of an unlabeled amino acid reduced the uptake of the same labeled amino acid in this experiment to less than 15%. For measuring the radioactivity trapped in the cells, the cells were lysed in 1 ml 1% Triton-X100 and radioactivity was measured in a scintillation counter. A statistically significant reduction of the amino acid uptake of more than 10% compared to the control was considered as a clear inhibitory effect.

The quality control of cell monolayers used in the permeability assays was conducted by measuring the transepithelial electrical resistance (TEER) of each monolayer using an epithelial voltameter (Millipore Corporation, MA, USA). Monolayers with TEER values in the 450–650 Ohm/cm^2 range were included in the permeability study. Furthermore the apparent permeability index (P_{app}) of ^{3}H-mannitol (paracellular marker, $P_{app} < 2 \cdot 10^{-6}$ cm/s) was computed. Permeability assays in apical-to-basolateral direction were conducted with CA at a concentration of 5 mM.

Results

CA can be released from proteins by proteolytic degradation

In order to test whether the non-protein amino acid CA can be liberated from proteins we alkylated soybean trypsin inhibitor (SBTI) with acrylamide and digested the alkylated protein with a series of proteases (pepsin, trypsin, chymotrypsin, elastase, and carboxypeptidase A). The digest of alkylated SBTI was analyzed by ESI-MS (Fig. 1). As indicated in the figure we found a peak at *m/z* 193 [M + H$^+$] that corresponds to the molecular weight of CA. Hence, we are confident that CA can be released from proteins by proteolytic degradation *in vitro.*

CA passes through Caco-2 cell monolayers

In order to test whether the non-protein amino acid CA can pass through Caco-2 cell monolayers, we measured the uptake of ^{35}S-CA from the apical to the basal compartment of Caco-2 cells. We found that ^{35}S-CA passes through the cells with an

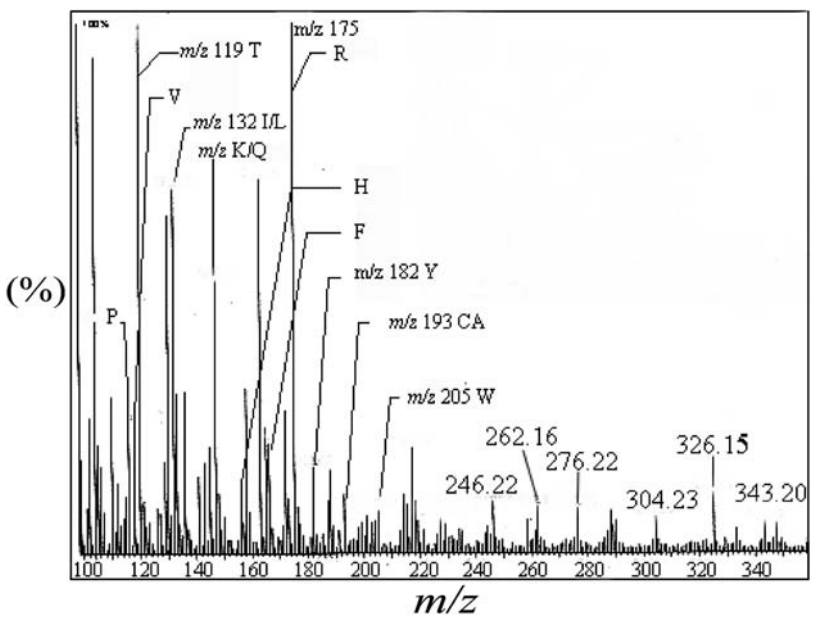

Fig. 1. ESI-mass spectrum of digested soybean trypsin inhibitor. Soybean inhibitor, alkylated by acrylamide, was digested with a set of proteases and analyzed by ESI-MS (positive mode). CA was identified as a peak at *m/z* 193 [M + H$^+$]. Amino acids others than CA detected in the spectrum are annotated with the single letter code.

apparent permeability of $(3.3 \cdot 10^{-4} \pm 5.5 \cdot 10^{-5})$ cm/s (n = 8). Since CA might be modified in the apical compartment (for instance hydrolysis) we also confirmed the uptake of intact CA from the apical compartment by ESI-MS (detection of *m/z* 193 $[M + H^+]$ for CA).

CA inhibits the uptake of proteinogenic amino acids

CA is structurally related to the amino acids asparagine and glutamine. Hence, we expected that CA interferes with the uptake of these amino acids in Caco-2 cells. In order to test this hypothesis, we measured the uptake of ^{3}H-labeled amino acids in Caco-2 cells in the presence and absence of a surplus of CA. As shown in Table I, CA does not interfere with the uptake of alanine, proline and glutamic acid, whereas in the presence of CA the uptake of arginine (Arg), histidine, leucine and glutamine is reduced by 20–40%. CA particularly inhibits the arginine uptake and hence we focused on this system in our further studies. In the next experiment we used various concentrations of CA and we found that inhibition of the arginine uptake is dose-dependent (Fig. 2).

In order to rule out that the observed transport inhibition is due to toxic effects of CA we tested different concentrations of CA in MTT and neutral red assays. Both, the MTT and neutral red assay showed that CA, in concentrations between 0.1 and 5 mM, is not toxic for Caco-2 cells. Furthermore, in order to exclude that CA is as a suicide substrate, we tested whether CA inhibition is reversible. Caco-2 cell monolayers were incubation with 1 mM CA for 30 min. The CA-containing compartment was discarded and the cells were intensively washed with Krebs-Ringer buffer. ^{3}H-labeled amino acid transport studies were conducted as de-

Table I. Inhibition of amino acid uptake by CA. 10 nM ^{3}H-labeled amino acids and 1 mM CA were applied on Caco-2 cell monolayers and incubated for 45 min. Radioactivity in the basolateral compartment was measured with a scintillation counter. Uptake refers to the uptake in the presence of CA versus the uptake in the absence of CA in percent.

Amino acid	Uptake (%) in the presence of CA	Transport systems
Alanine	91 ± 7	Neutral, B^{0+}, y^+L, b^{0+}
Arginine	65 ± 7	Cationic, B^{0+}, y^+L, b^{0+}, y^+
Histidine	58 ± 5	Neutral, cationic
Glutamine	79 ± 6	Neutral, B^{0+}, y^+L, b^{0+}
Proline	110 ± 3	Imino, A
Glutamic acid	94 ± 7	X^-_G, x^-
Leucine	64 ± 2	Neutral, L, B^{0+}, y^+L, b^{0+}

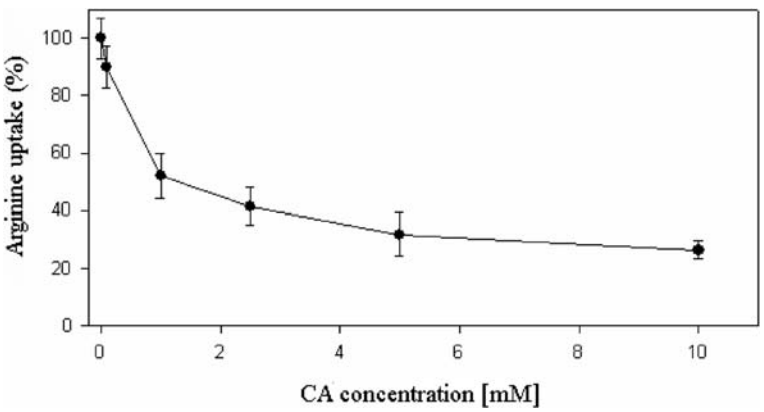

Fig. 2. Dose-dependent inhibition of the arginine uptake by CA. 10 nM ^{3}H-arginine and various CA concentrations (0–10 mM) were applied on the apical side of Caco-2 cell monolayers, seeded on Transwell polyester membrane filters. After 45 min of incubation, the radioactivity in the basolateral compartment was measured in a scintillation counter.

scribed above. CA pre-incubated Caco-2 cell monolayers showed no statistically significant reduced arginine transport compared to untreated cells ($p = 2.7 \cdot 10^{-4}$; $n = 6$; DF = 5). Similar results were achieved for other amino acids. Hence, we conclude that CA interferes with the uptake through the neutral and cationic amino acid transport systems in a reversible and dose-dependent manner.

Inhibition of amino acid transport is competitive

The kinetics of arginine uptake is a combination of active transport described by the Michaelis-Menten kinetics and passive diffusion described by Fick's law. Therefore, K_m and J_{max} were calculated for the uptake of arginine in the presence of CA according to the formula: $v = v_{max} \cdot S/(K_m + S) + K \cdot S$ by non-linear regression. In the presence of CA, the K_m value of the arginine uptake increased by the factor 8.9 and the value of J_{max} by the factor 1.6 (Table II). This data demonstrate that CA inhibits the arginine uptake in a competitive manner.

Discussion

Acrylamide covalently binds to proteins, in particular to cysteine residues, under alkaline conditions. Due to the alkaline pH in the gut, acrylamide can react with dietary proteins, peptides or cysteine, before it passes the intestinal barrier. The further fate of protein-bound acrylamide, however, is unknown. Recently, Bjellaas *et al.* (2007) reported that the majority of orally administered acrylamide is excreted as NAPC. Protein-bound acrylamide, however does not pass through Caco-2 cells and, therefore, it is unlikely that it will contribute to the level of NAPC in the urine. These data indicate that either protein-binding of acrylamide in the intestine occurs to a very low extent or protein-bound acrylamide can be liberated in the form of CA (the precursor of NAPC) in the intestine, and then appear as NAPC in the urine.

In the intestine, proteins are enzymatically degraded to amino acids, di- and tripeptides. Using a set of proteases, that naturally occur in the intestine, we simulated protein digestion *in vitro*. The ESI-MS spectrum of the digest showed that a molecule of the molecular weight of CA can be released from proteins via digestion with gastro-intestinal proteases *in vitro*. In fact, pepsin, elastase and carboxypeptidases show very little substrate specificity when the substrate protein is fully denaturated. Hence, we expect that CA can also be released from alkylated proteins *in vivo*.

We then demonstrated that radioactive CA can pass Caco-2 cell monolayers and that non-radioactive CA inhibits the uptake of amino acids by the neutral and cationic amino acids transport systems. The major target for CA is the arginine transport system, which is inhibited in a dose-dependent, reversible and specific manner. The uptake of arginine is catalyzed by two distinct cationic amino acid transport systems: a sodium-dependent (B^{0+}, y^+L) and a sodium-independent (b^{0+}, y^+) system (Pan *et al.*, 1995a). Indeed we found that the uptake of CA is partially Na^+-dependent (data not shown) and that CA supplement alters the K_m value, but not the J_{max} value of the arginine uptake. These data show that CA is a competitive inhibitor for the arginine transporters. Competitive inhibitors compete against the substrate for the catalytic pocket and therefore we conclude that CA is a potential substrate itself. Furthermore, taken together with the data that CA, although it is a hydrophilic molecule, can pass the monolayer, we conclude that CA is indeed a substrate for the neutral and cationic amino acid transport system and consequently is transported through Caco-2 cells.

Caco-2 cells are a common model to study the intestinal amino acid uptake (Pan *et al.*, 1995b), and it is likely that CA can follow the same route in the human intestine. Since the human *N*-acetyltransferases 1 and 2 (EC 2.3.1.5) are expressed in the epithelium of the intestine, colon and the urinary bladder, it is further reasonable that CA will be *N*-acetylated, similar to CA generated from glutathione (Windmill *et al.*, 2000). Consequently, CA formed in the gut may be excreted as NAPC with the urine.

Table II. Michaelis-Menten parameters of the arginine transport system. Caco-2 cell monolayers were incubated with arginine (Arg, 0.1–4 mM) and the uptake was measured after 45 min. CA concentration in the inhibitor experiment was 1 mM. The parameters were calculated by nonlinear regression according to: $J = [J_{max} \cdot S/(K_m + S)] + K \cdot S$. Both data sets are significant (Arg: $R^2 = 0.996$; Arg + CA: $R^2 = 0.998$; DF = 9).

	J_{max} [mol/min]	K_m [μM]
Arg	$(7.1 \pm 1.2) \cdot 10^{-11}$	54 ± 24
Arg + CA	$(11.4 \pm 2.7) \cdot 10^{-11}$	476 ± 184

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