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Reduction of Acrylamide Uptake by Dietary Proteins in a Caco-2 Gut Model

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The report of elevated acrylamide levels in some foods raised an international health alarm, because acrylamide probably has carcinogenic, neurotoxic, and genotoxic properties. However, data on the bioavailability of acrylamide from food matrices in humans are limited. In particular, only little is known about the interactions of acrylamide with food ingredients. Using a human intestine model (Caco-2 cells), this study shows that acrylamide monomers are highly bioavailable and pass the cell monolayer via passive diffusion. Furthermore, acrylamide binds to dietary proteins such as chicken egg albumin under intestinal and cooking conditions. This binding reduces the concentration of acrylamide monomers and leads to a reduced uptake by Caco-2 cells. Hence, it is concluded that a protein-rich diet may reduce acrylamide uptake.

KEYWORDS: Caco-2 cells; acrylamide; bioavailability; intoxication; toxin exposure

INTRODUCTION

The recent report of elevated acrylamide levels in certain food items evoked an international health alarm. The occurrence of acrylamide levels of up to 4000 μg/kg (*1*), formed by the Maillard reaction (*2*, *3*) raised much concern, because acrylamide shows carcinogenic properties (*4*, *5*) and toxic effects on the reproductive (*6*, *7*) and nervous systems (*4*, *5*, *8*, *9*). Neurotoxicity has been demonstrated in humans (*10*−*12*). Although in these studies the uptake of acrylamide was through routes of exposure other than ingestion of endogenously formed acrylamide in foods, dietary intake of acrylamide is of general public concern.

A prerequisite for the toxic effects of acrylamide is its uptake into the body. Animal-feeding experiments reveal a rapid and complete absorption after oral administration in mammals (*13*−*17*). Whether these data reflect human exposure remains unclear because acrylamide in animal experiments is usually supplied with the drinking water (*4*−*6*, *17*). Acrylamide is a reactive molecule, and therefore interactions with food ingredients are likely. However, little has been done to investigate the fate of acrylamide in the intestine.

In this study, we employed the well-established transwell filter chamber system containing Caco-2 cells to study the passage of acrylamide through the gastrointestinal epithelium. Originating from a human adenocarcinoma, Caco-2 cells differentiate into a monolayer of different types of polarized intestinal cells when seeded on microfilters and exhibit many properties typical of a permeable gut epithelium (*18*, *19*).

Our results show that acrylamide monomers pass the intestinal cell monolayer via passive diffusion, and therefore a high bioavailability can be expected (*20*, *21*). However, we further show that incubation of acrylamide monomers under intestinal conditions with a cysteine source leads to a significant reduction of free acrylamide. This assumption is also true for protein with cysteine residues. Forming covalent bonds with dietary proteins reduces the concentration of free acrylamide and leads to a reduced uptake. Hence, we conclude that a protein-rich diet will reduce the uptake of acrylamide.

EXPERIMENTAL PROCEDURES

Cell Culture. The established human epithelial Caco-2 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies), containing 20% fetal bovine serum (FBS, Biochrom KG). The medium was additionally supplied with 1 mM sodium pyruvate (Biochrom KG), 10 mM nonessential amino acids (Biochrom KG), 2 mM l-glutamine (Gibco Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Biowittaker Europe). Cells were cultured in 75 cm^2 flasks (Greiner Bio-one) and incubated at 37 °C with 5% CO_2 at 90% relative humidity. Culture media were changed every second day.

Permeability Assay. Cells were washed two times with Hanks buffer salt solution without Ca^{2+} or Mg^{2+} and then harvested from the flasks with a trypsin−EDTA solution (0.25−0.02% w/v without Ca^{2+} or Mg^{2+}, Gibco Life Technologies) and seeded at a density of 60000 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)] 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 glucose. Cell monolayers were used between 14 and 21 days and between 21 and 28 days postseeding for uptake and permeability studies, respectively.

Bidirectional permeability (apical-to-basolateral and basolateral-to-apical) experiments were conducted with acrylamide at concentrations

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of 10 and 1 mM. The solutions were added to the chamber facing the apical or basolateral surface of the cells, respectively. The uptake was then determined every 15 min for 90 min in samples taken from the opposite chamber. At each time point 100 μL was removed from the receiver chamber for measurement and replaced with fresh assay buffer. Each experiment was replicated at least three times.

Inhibition of cell energy metabolism was used to determine if acrylamide permeates the cells by active transport or passive diffusion. Cells were preincubated with 2-deoxyglucose (100 mM), an inhibitor of glycolysis, or at low temperatures (4 °C) for an appropriate time before and during the experiment.

The quality of cell monolayers employed in transport assays was controlled by measuring the transepithelial electrical resistance (TEER) of each monolayer using an epithelial voltameter (Millipore Corp.). Monolayers with TEER values in the 400−650 ohm cm^2 range were included in the permeability study. Furthermore, the apparent permeability index (Papp) of a paracellular marker ([^{3}H]mannitol, Papp $< 2 \times 10^{-6}$ cm/s) was determined. To estimate whether the applied acrylamide concentrations would harm the cells, [^{3}H]arginine (1 mM) uptake activity was used as an indicator of cell damage after acrylamide exposure. Treatment of Caco-2 cells with acrylamide in concentrations between 1 and 10 mM leads to a small reduced [^{3}H]arginine uptake (<20%). Furthermore, a 1 h exposure of 1−10 mM acrylamide to Caco-2 cells revealed no cytotoxity in an MTT assay, a quantitative colorimetric method for the determination of cell survival and proliferation (*22*) (data not shown).

Permeability Calculation. The apparent permeability (Papp) was calculated according to the equation

$$\text{Papp} = (\mathrm{d}C_r/\mathrm{d}t) \times V_r/(AC_0)$$

where $\mathrm{d}C_r/\mathrm{d}t$ is the cumulative concentration in the receiver compartment versus time given in mol L^{-1} s^{-1}. $\mathrm{d}C_r/\mathrm{d}t$ was determined by linear fit (without origin). In general, the correlation coefficients exceed 0.90. V_r is the volume of the receiver compartment ($a = 0.5$, bl = 1.5 cm^3). A is the area of the cell monolayer (1.13 cm^2 for a 12-well Transwell), and C_0 is the concentration of the dosing solution in mol L^{-1}.

Analysis of Acrylamide. Quantification of acrylamide was performed by reversed phase HPLC (RP-HPLC) using a Beckman system gold HPLC with an 18 cm Lichrospher 100 RP18 column. The HPLC was operated isocratically at a flow rate of 1 mL/min. Water was used as mobile phase. Injection volume was 30 μL. Transport of acrylamide through Caco-2 cell monolayers was confirmed using a VG Quattro II electrospray ionization tandem mass spectrometer (ESI-MS) (Micromass, U.K., Ltd., Altrincham, Cheshire, U.K.). The ESI-MS was operated in the positive electrospray mode (U = 3.5 kV). Samples were applied in a concentration of 1 ng/μL in 50% methanol and 2% formic acid with an A99 syringe pump (Razel, Stamford, CT) at a flow rate of 35 μL/min. Acrylamide was detected as a single peak with an m/z ratio of 72 ($M + H^+$) (*23*). In transport experiments the acrylamide concentration was quantified by UV spectrometry at 230 nm (linear range 6−1000 μM, $R^2 = 1$).

Acylamide Binding Studies. The binding properties of acrylamide to cysteine residues were investigated using glutathione in an HPLC assay. Glutathione and acrylamide were incubated in a molar ratio of 5:1 in phosphate-buffered solutions prepared to meet the pH in the gastrointestinal tract (pH 6−8). Reaction time was 45 min at 37 °C. The reaction solutions were directly applied to the RP-HPLC. Binding of acrylamide (1 mM) to dietary proteins was investigated by incubation of acrylamide in a 5% (w/v) solution of a chicken egg albumin (Merck, Darmstadt, Germany) at two temperatures (37 and 80 °C). Samples were taken every 20 min. Proteins were precipitated by the addition of 2-propanol and removed by centrifugation. The acrylamide containing supernatant was analyzed by RP-HPLC.

Statistics. Statistical analysis was performed using Student's t test. In all experiments, values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Acrylamide Passes Caco-2 Cells by Passive Diffusion. Because acrylamide is a small polar molecule, we expected Caco-2 cell monolayers to be permeable to acrylamide. The monolayers were incubated either apically or basolaterally with acrylamide to study transport in both directions. Permeation of acrylamide across cells was confirmed by ESI-MS and quantified spectrometrically.

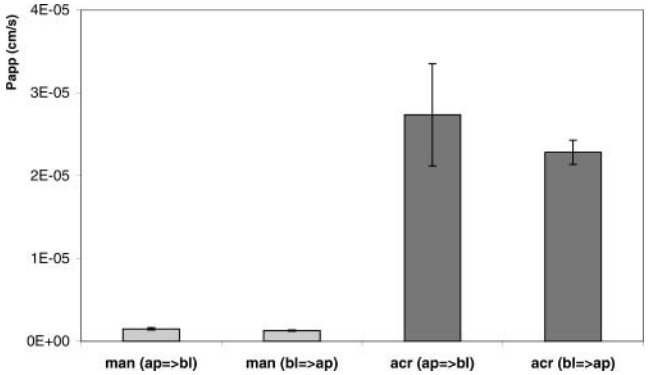

Figure 1. Caco-2 cell monolayers are permeable to acrylamide (acr). The calculated Papp value for apically to basolaterally (ap → bl) uptake is $2.7 \times 10^{-5} \pm 6.2 \times 10^{-6}$. Results for basolateral to apical (bl → ap) passage (Papp $2.3 \times 10^{-5} \pm 1.5 \times 10^{-6}$) were not statistically different ($t = 1.8$, $p = 0.11$, df = 10) from ap → bl. Both Papp values indicate fast passage of acrylamide through the monolayer. For comparison, mannitol (man) as a paracellular marker does not pass through the monolayer to a high degree. Error bars represent standard deviation. Acrylamide was detected on the basolateral side of the monolayer by ESI-MS and quantified spectrometrically.

The apparent permeability coefficients (Papp) for both directions apically to basolaterally (ap → bl), and basolaterally to apically (bl → ap) are shown in **Figure 1**. We did not observe a statistically significant difference in the Papp values for the transport ap → bl and bl → ap. A statistically significant difference would be expected in the case of an active transport through the monolayer in one direction.

Inhibition of glycolysis with 2-deoxyglucose decreases statistically significantly the active, ATP-dependent transport of amino acids but has no effect on acrylamide uptake ($t = -0.95$, $p = 0.40$, df = 4). Passive diffusion of acrylamide through Caco-2 cell monolayers was further confirmed by incubation at low temperatures. In our transport studies conducted at 4 °C glutamine transport was reduced to 30% of the value at room temperature, wheras acrylamide uptake was reduced to <50%. These data are in agreement with the observation that passive diffusion is less sesitive to temperature changes than active transport systems (*24*).

Although most transporters exhibit substrate specificity, some of them accept molecules structurally related to the substrate. The closest structural relative of acrylamide that is actively transported through the intestine is urea. The recently identified urea transporters, however, are catalyzing the transport of urea from the basolateral to the apical direction (*25*). MacAuley et al. reported that urea passively permeates cell membranes via the sodium glutamate cotransporter (*26*). Therefore, we tested if acrylamide interacts with glutamate uptake in Caco-2 cells. No inhibition of glutamate transport through Caco-2 cells by acrylamide was observed using radioactively labeled glutamate even at high acrylamide concentrations (2 mM).

In conclusion, these results indicate that acrylamide readily passes in both directions through the Caco-2 cell monolayer via passive diffusion.

The diffusion-driven uptake of acrylamide could be via either the paracellular or the transcellular pathway. Tight junctions connecting the cells do not effectively restrict the passage of

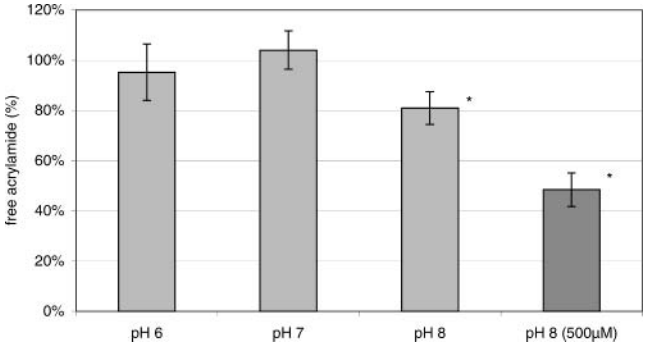

Figure 2. pH dependency of the condensation of acrylamide with glutathione. Fifty micromolar acrylamide was incubated together with 250 μM glutathione for 45 min at 37 °C at different pH values. Whereas reaction at acidic (pH 6) and neutral (pH 7) pH reveals no significant reduction of acrylamide monomers compared to a 1 mM acrylamide solution, reaction under alkaline conditions (pH 8) reduces the amount of acrylamide monomers to 81% ($t = 7.6$, $p < 0.001$, df = 30). An increased glutathione concentration (500 μM) further reduces the concentration of acrylamide monomers to 48% ($t = 13.7$, $p < 0.001$, df = 19). Error bars represent standard deviation. * = $p < 0.05$. Acrylamide was quantified by RP-HPLC.

molecules with a molecular weight (MW) < 150 (*27*) (MW acrylamide = 71). Nevertheless, an additional transcellular pathway cannot be excluded, because other small polar molecules such as urea and glycerol have been reported to diffuse across protein-free lipid bilayers (*28*).

Our data further indicate that acrylamide cannot be detoxified via active efflux pumps (ABC transporters) that are present in Caco-2 cells (*18*). Otherwise, a higher basolateral to apical Papp value as well as an influence of physiological inhibitors (2-deoxyglucose) would be expected.

The calculated Papp can be used to classify acrylamide as a compound with a high bioavailability in humans (*18*, *21*). Hence, our results are in agreement with the observed rapid and complete absorption of acrylamide from the intestinal tract in feeding experiments (*13*−*17*).

Acrylamide Binds to Dietary Proteins under Intestinal Conditions. Although acrylamide monomers readily diffuse through Caco-2 monolayers, acrylamide uptake from food in the human intestine may differ from these experimental conditions. Acrylamide contains a reactive terminal double bond, which may interact with food ingredients, mainly proteins, DNA, and RNA.

Because thiols are the most reactive groups in proteins to which acrylamide might bind under physiological conditions, we tested whether acrylamide reacts with glutathione, a small peptide containing one cysteine residue with a reactive SH group. In vitro incubation of acrylamide together with glutathione at different intestinal pH values and at body temperature revealed no statistically significant reduction of acrylamide monomers at acidic and neutral pH values. However, incubation at pH 8 statistically significantly reduces the amount of acrylamide monomers to 81% (**Figure 2**) as expected for a Michael addition like mechanism, which is favored at alkaline pH values.

A higher availability of cysteines (molar ratio 1:10; acrylamide/glutathione) led to an increased reduction of acrylamide monomers (48%) (**Figure 2**). We consider that acrylamide is most likely bound covalently to glutathione via Michael addition of cysteine residues to the reactive terminal double bond (*29*, *30*). However, also a binding to the primary amino group cannot be excluded.

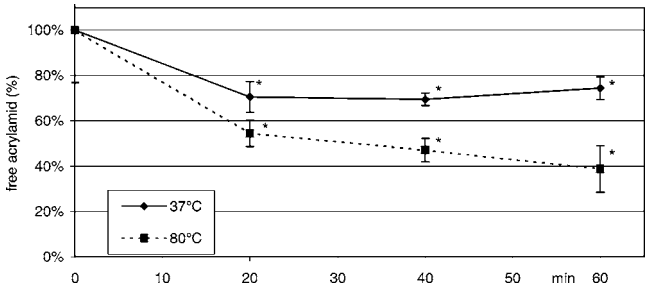

Figure 3. Binding kinetics of acrylamide to dietary proteins. Incubation of 1 mM acrylamide in a 5% solution of chicken egg albumin leads to a reduction of acrylamide monomers to 75% during the first 20 min. Increased reaction times do not lead to further binding. Increased temperature (80 °C) further reduce acrylamide monomers. No endpoint of the reaction was observed after 60 min at 80 °C. Acrylamide was quantified by RP-HPLC after protein precipitation with 2-propanol. Error bars represent standard deviation. * = $p < 0.05$.

Because glutathione is a model peptide, we mimicked the situation in the gut more closely by incubating acrylamide together with a dietary protein. In vitro incubation of acrylamide (1 mM) in a 5% solution of a chicken egg albumin under alkaline conditions (pH 8) revealed reductions to 75% (37 °C) and 50% (80 °C) of free acrylamide (**Figure 3**).

We conclude that acrylamide can bind to dietary proteins under physiological conditions in the intestine or under mild cooking conditions (80 °C). The difference between the results at 80 and 37 °C may be due to the higher reaction rate at higher temperatures. Furthermore, cleavage of disulfide bonds at 80 °C would provide an increased number of cysteine residues (*31*).

Binding of Acrylamide to Proteins Reduces the Acrylamide Uptake through Caco-2 Cell Monolayers. Because passive diffusion is concentration dependent, we expected a reduced passage of acrylamide through Caco-2 cells in the presence of dietary proteins. Therefore, we applied a 1 mM acrylamide solution preincubated in a 5% solution of a chicken egg albumin at 37 and 80 °C (pH 7.4) onto the apical side of Caco-2 cell monolayers. Compared to a control of 1 mM acrylamide the transport of acrylamide preincubated in a protein solution was reduced to 80% (37 °C) and 59% (80 °C) (**Figure 4**). This result indicates that the binding of acrylamide to proteins reduces the absorption of acrylamide by Caco-2 cells. This experiment shows further that reduction of free acrylamide already occurs under mild alkaline conditions. Because proteins contain more reactive groups than glutathione, binding to other groups such as amino or hydroxyl groups may also be possible.

Binding of acrylamide to amino acid residues of dietary proteins has potential consequences in vivo. Because the reaction of acrylamide with cysteine residues is favored only at alkaline pH values, the in vivo binding will mainly occur in the distal jejunum, ileum, and colon, where intestinal pH can reach values between 7 and 8 (*32*). Hence, it seems very likely that under the pH regime of the small intestine a reduction of free acrylamide during gut passage is possible. Additionally, a reduction of free acrylamide during food preparation can be assumed.

In our study an incubation of 1 mM acrylamide in a 5% protein solution results in a reduction of free acrylamide to 80%. However, acrylamide levels in food rarely exceed 3000 μg/kg. Therefore, an assumed uptake of 3.0 mg of acrylamide (0.04 mmol) per kilogram food in a meal with a common protein content of >10% would result in a much higher ratio of protein to acrylamide. Therefore, we expect much higher acrylamide binding in vivo.

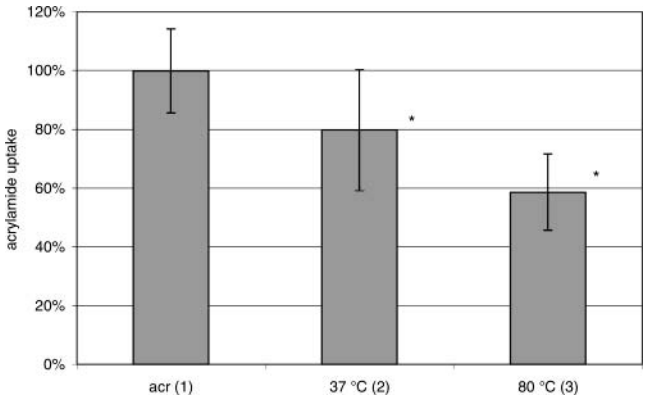

Figure 4. Reduced uptake of acrylamide by Caco-2 cells in the presence of dietary proteins. Preincubation of 1 mM acrylamide in a 5% dietary protein solution for 45 min at 37 °C (pH 7.4) leads to a reduced uptake of acrylamide by Caco-2 cells (2) compared to an untreated acrylamide solution (1 mM) (1). Further reduction can be achieved by preincubation at 80 °C (3). Acrylamide was applied onto the apical side of the Caco-2 monolayer. Basolateral acrylamide concentration was measured spectrometricaly at 230 nm over 60 min. Columns represent the mean uptake over 60 min. Error bars represent standard deviation. * = $p < 0.05$.

Although acrylamide monomers exhibit a high bioavailability, the bound form cannot pass the Caco-2 cell monolayer by diffusion. However, proteins with bound acrylamide most likely will undergo common protein digestion. The fate of the acrylamide-conjugated cysteines in the intestine has not been investigated.

If alkylated cysteines are released after protease processing in the gut and absorbed in the intestine, we expect them to be detoxified by the common mercapturic acid pathway. In rodents, most of the administered acrylamide is detoxified by this pathway (*33*, *34*). After binding a toxin to the glutathione cysteine residue, the modified cysteine is released by the γ-glutamate transpeptidase and cysteine glycinase. Because the acrylamide-conjugated cysteine resembles the modified cysteines from the glutathione detoxification pathway, we expect the acrylamide-conjugated cysteine to be N-acetylated and eliminated by the kidney.

In summary, we conclude that high protein concentrations in the human diet may reduce acrylamide uptake.

ABBREVIATIONS USED

Caco-2, colon adenocarcinoma cell line; Papp, apparent permeability index; TEER, transepithelial electrical resistance; MTT, methylthiazolyldiphenyltetrazolium bromide; ESI, electrospray ionization; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MW, molecular weight; RP, reversed phase; acr, acrylamide; man, mannitol; ap, apical; bl, basolateral.

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