IN VITRO AND ANIMAL STUDIES

Investigation of the effects of soluble fibers on the absorption of resveratrol and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP) in the Caco-2 cellular model of intestinal absorption

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Abstract
Soluble fibers are known to modulate intestinal absorption of non-polar compounds in the small intestine. Little is known about the modulation of absorption of more polar compounds. In the present study, we applied the Caco-2-transwell-system in order to investigate the modulation of intestinal bioavailability by soluble fibers. The system was tested using pectin and carrageenan as model soluble fibers at a concentration of 0.1% (w/v), which did not compromise the integrity of the cell monolayer. Modulation of absorption was evaluated for the heterocyclic amine aromatic 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP) and the polyphenol resveratrol. Neither pectin nor carrageenan reduced the high flux of PHIP, apparent permeability coefficient (Papp) of 16 × 10−6 cm s−1. The low Papp of resveratrol was reduced by both soluble fibers, particularly by pectin. These results suggest that the low bioavailability of polyphenols could be further reduced by soluble fibers. Because of their co-occurrence in several fruits, these findings warrant further research.

Keywords
Heterocyclic aromatic amines Caco-2 transwell system, intestinal absorption, polyphenols, resveratrol, soluble fibers

Introduction
The beneficial effects of fibers on human health are widely accepted. Several expert associations recommend a substantial intake of fibers, e.g. the German Society for nutrition Deutsche Gesellschaft für Ernährung (DGE) recommendation is 30 g fiber each day (DGE, 2000). A significant portion of these compounds are soluble – non-starch polymeric carbohydrates – which can be fermented by the human gut bacteria (Anderson et al., 2009). The fermentation products, particularly short chain (fatty) acids, are absorbed and used by the human body as a source of energy. Thus, for the estimation of the energy content of food 1 g of fibers accounts for 1 kcal. Because the short chain fatty acids act anti-inflammatory, they reduce the risk for colon cancer (Rose et al., 2007). Moreover, they non-covalently bind non-polar compounds in the small intestine and reduce their absorption, as described, e.g. for drugs like digitoxin (Fugh-Berman, 2000). Based on the same mode of action, soluble fibers, e.g. the β-glucans from oat, reduce the reabsorption of bile acids which could be beneficial in case of hypercholesterolemia (Braaten et al., 1994). The standard in vitro tool for the investigation of oral bioavailability of drugs is the Caco-2 transwell system (Hubatsch et al., 2007). In the present study, we explored if this system is a suitable model for the investigation of the modulation of absorption of bioactive medium-polar food ingredients. In this explorative study, we investigated the effects of the pectin (α-(1–4) linked β-d-galacturonic acid with modifications) and carrageenan (mixture of α-1,3 and β-1,4 linked galactose anhydrogalactose and other saccharides containing three sulfates per disaccharide unit) on the absorption of two polar compounds, the toxic heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP) and the polyphenol resveratrol.

Materials and methods

Chemicals and biological materials
Caco-2 cells were purchased from the American Type Culture Collection (ATCC; distribution by LCG, Wesel, Germany). Pectin from apple (Sigma 76282), λ-carrageenan (Sigma 22049) trans-resveratrol (≥99% purity), β-glucuronidase (GUS) from Helix pomatia (HP-2 specific activity: GUS ≥ 100 000 U mL−1, sulfatase: ≤7,500 U mL−1) and all other chemicals were obtained from Sigma Aldrich (Schnelldorf, Germany). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and all cell culture reagents were purchased from Biochrom (Berlin, Germany).

Caco-2 system
Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids, 2 mM glutamine and 100 IU mL−1 penicillin and 100 μg mL−1 streptomycin. For the...
transport experiment, the sub-confluent cells were seeded at a density of \(1 \times 10^5\) cells/well in inserts ("ThinCerts", Greiner Bio One, Frickenhausen, Germany) with 0.5 mL of medium in the apical compartment and 1.5 mL medium in the basolateral compartment. The medium was changed three times a week. The transport experiments were performed 23–30 d post-seeding in DMEM medium adjusted to pH 7.4 containing 50 mM TRIS buffer and without added phenol red or FBS. The integrity of the Caco-2 cell monolayer was assayed by using only wells exceeding a trans-epithelial electrical resistance (TEER) of 300 \(\Omega\) cm\(^{-2}\) before and after treatment with the test compounds. Experiments were carried out with and without pectin or carrageenan (0.1% w/v) with a concentration of 20 \(\mu\)M of resveratrol and 5 \(\mu\)M PHIP. After 3 h, 60 \(\mu\)L of each well were taken as samples from the basolateral side and 20 \(\mu\)L from the apical side. After the last sampling point (6 h), the whole medium was collected from both chambers and the cells were lysed by incubation with 1% Triton\(^{X-100}\) solution for 10 min. In order to assure that the integrity of the monolayer is not affected by resveratrol, PHIP as well as pectin and carrageenan, the transport experiments were performed additionally in the presence of Lucifer yellow (LY, 100 \(\mu\)M in the apical compartment). After 6 h of incubation, LY could be detected in the basolateral chamber, indicating that the polyphenols did not compromise the integrity of the monolayer. In order to liberate polyphenols bound as conjugates, sample medium collected of both compartments was additionally incubated over night with 1000 \(\mu\)M GUS. The apparent permeability coefficient (\(P_{app}\)) was calculated according to Formula 1.

**Formula 1:** Calculation of the apparent permeability coefficient according to Artursson & Karlsson (1991).

\[
P_{app} = \frac{\Delta c \times V}{\Delta t \times c_0 \times A} \text{ cm} \quad \text{s}^{-1} \quad \text{M}
\]

\(\Delta c\) is the concentration (\(\mu\)M) in the receiver compartment

\(V\) is the volume of the receiver compartment

\(\Delta t\) is the duration of the transport experiment in seconds

\(c_0\) is the initial concentration in the donor compartment in \(\mu\)M

\(A\) is the surface area of the membrane (cm\(^2\)).

**Quantitative analysis**

Quantification of resveratrol and PHIP was carried out by liquid chromatography (LC) with automated sample preparation by online-solid phase extraction (SPE) in back flush mode as described (Willenberg et al., 2014, 2015). In brief, resveratrol was analyzed by online-SPE-LC with ultraviolet (UV) absorbance detection using formononetin as internal standard (IS) (Willenberg et al., 2014). The limit of quantification was 0.2 \(\mu\)M and the accuracy and precision of the method was not affected by the fibers (Table S1). Quantification of PHIP was carried out with tandem mass spectrometry (MS/MS) detection in selected reaction monitoring mode utilizing PHP-d3 as stable isotope-labeled IS.

**Results**

As shown in Figure 1, PHIP showed a significant flux through the Caco-2 monolayer. The resulting \(P_{app}\) without addition of fibers was in same range as previously reported (Willenberg et al., 2014, 2015). Addition of carrageenan and pectin at a concentration of 0.1% (w/v) led to no increase of LDH activity (data not shown) in the medium indicating no cytotoxicity. Moreover, the TEER remained over 300 \(\Omega\) cm\(^{-2}\) after incubation indicating integrity of the cell monolayer. For PHIP, no differences were found in the distribution between apical and basolateral chamber (Figure 1). Accordingly, the resulting \(P_{app}\) was not changed: control: 16 ± 3.8 \(10^{-6}\) cm s\(^{-1}\) pectin 17 ± 2.9 \(10^{-6}\) cm s\(^{-1}\) and carrageenan 16 ± 1.3 \(10^{-6}\) cm s\(^{-1}\). For resveratrol, the same overall pattern of free and conjugated (glucuronidated) compound on the apical and basolateral side was observed, with a slightly lower overall recovery for the soluble fibers (Figure 1). However, the amount of free resveratrol reaching the basolateral side after 6 h was reduced by pectine and carrageenan leading to a lower \(P_{app}\) (Figure 1, Table 1). Because of the low concentration of resveratrol detected on the basolateral side, the variation in \(P_{app}\) in particular in presence of carrageenan was considerably high. However, similar effects of the fibers on total (free+conjugated) resveratrol in the basolateral chamber were observed (Figure 1) indicating a reduced absorption.

**Discussion**

In this study, the standard model for in vitro evaluation of intestinal absorption was applied on the investigation of modulation of food ingredients. Earlier studies utilizing this approach focused on the absorption of non-polar compounds, such as carotenoids (Yonekura & Nagao, 2009). For these compounds, being absorbed together with lipids, the soluble fibers led to an
inhibition of micellization, thus reducing the cellular uptake in the Caco-2 model.

In our study, we focused on two (medium) polar food ingredients, PHIP and resveratrol. With a low-molecular weight, a good water solubility and a Log n-octanol/water coefficient of ~2 (Log P), both compounds, could be absorbed by diffusion directly from the gut lumen.

For the model compound of the heterocyclic amines, PHIP [Log P = 1.78 (Borosky, 2008)], no influence of the soluble fibers on the flux through the cells was observed. This indicates that there is no or little (non-covalent) binding of PHIP to the polysaccharides and the effective concentration in the medium is not affected. Thus, the diffusion through the Caco-2 cell layer is not affected. For the situation in vivo, these results indicate that the high oral bioavailability of the potentially carcinogenic PHIP (Nicken et al., 2015) is not affected by soluble fibers. These results are somewhat conflicting with other studies concluding for heterocyclic aromatic amines (HAAs) that soluble and none-soluble fibers could reduce bioavailability of non-covalent binding (Raman et al., 2013; Sjödin et al., 1985). However, these studies are based on the quantification of the bound/free amount of the HAAs and did not include absorption through an intestinal barrier (model). On the other hand, they used more realistic mixtures of fibers. Overall, the question whether fibers could lead to a biologically relevant reduction of the absorption of HAAs can only be answered based on the bioavailability studies in vivo.

The prominent potentially health-promoting polyphenol resveratrol was chosen as model compound for the class of polyphenols. With a Log P of 1.87 (Fabris et al., 2008), it is similarly polar as PHIP. Because of the rapid conjugation of the polyphenol by UGT present in Caco-2 cells (Willenberg et al., 2012, 2014), the vast majority of resveratrol was found in its conjugated form after incubation (Figure 1). When comparing both, the concentration of both free and free + conjugated resveratrol reaching the basolateral side, it becomes apparent that both soluble fibers reduce the flux through the Caco-2 cell monolayer (Table 1). Particularly, the low amount of unchanged resveratrol reaching the basolateral side is reduced almost by half. These effects are most probably caused by non-covalent binding of resveratrol to the soluble fibers. The resulting complex cannot pass the cell membrane and the concentration of resveratrol is reduced leading to a lower flux (D’Archivio et al., 2010).

Conclusion

For the heterocyclic amine, PHIP, no influence of pectin and carrageenan on the flux through the Caco-2 cells was observed. However, the amount of the polyphenol resveratrol reaching the basolateral side of the trans-well system was reduced by the fibers. Extrapolating these data to the situation in vivo, it could be concluded that the poor bioavailability of polyphenols (D’Archivio et al., 2010) may be further reduced by soluble fibers. As pectin and several other soluble fibers occur in the same plants as the polyphenols, these findings warrant further investigation.

Declaration of interest

This study was supported by the Institut Danone Ernährung für Gesundheit e.V. The authors declare that there are no conflicts of interest.

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Supplementary material available online

Supplementary Table S1