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Single and concerted effects of benzo[a]pyrene and flavonoids on the AhR and Nrf2-pathway in the human colon carcinoma cell line Caco-2

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ABSTRACT

As phytochemicals have the potential to counteract adverse effects of carcinogens we investigated the influence of the flavonoids quercetin and kaempferol on benzo[a]pyrene (BaP) mediated effects on human colon cancer cells, Caco-2. We focused on concerted effects on the expression of AhR and Nrf2 pathway components. In contrast to kaempferol, BaP and quercetin efficiently induced CYP1A1, CYP1A2 and CYP1B1-mRNA in Caco-2 cells. BaP not only acted via AhR activation but sustainably also by increasing AhR and by down-regulating AhRR mRNA. The flavonoids did not affect AhR expression but counteracted the BaP mediated AhRR repression. Only quercetin was found to induce AhRR mRNA. ARNT mRNA appeared to be slightly but significantly down-regulated by BaP as well as by flavonoids while expression of AIP was not or only slightly modulated. The Nrf2 pathway was activated by BaP and by the flavonoids shown by induction of Nrf2 and several of its target genes such as NQO1, GSTP1, GSTA1 and GCLC. Induction effects of 10 μm BaP on Nrf2, GSTP1 and NQO1 were abolished by the flavonoids. In summary, we show that quercetin supports AhR mediated effects. Both flavonoids, however, may counteract the effects of BaP on expression of AhR, AhRR, Nrf2, GSTP1 and NQO1. In conclusion, quercetin appears to have two faces, a flavonoid-like one and a PAH-like one which supports Ahr-mediated effects while kaempferol acts "just like a flavonoid". Thus, flavonoids have to be treated individually with respect to their anti-adverse activity.

1. Introduction

The human colon carcinoma cell line Caco-2 and its derivatives have been widely used in studies on molecular effects of and interactions with xenobiotics. In this respect, the cell line served as model system also for cells of the small intestine as it undergoes a differentiation during culture which results in an ileum cell like phenotype (Meunier et al., 1995; Sambuy et al., 2005). Particularly, membrane transport and metabolism of xenobiotics were studied (Fan et al., 2010; Pang et al., 2009) which both influence the final concentrations of ingested xenobiotics in the circulation and the body. Consequently, the intestine has a particular role in the primary defense against toxic compounds and carcinogens but is also a target of their adverse effects. Colorectal carcinomas are the third most common form of cancer and the second leading cause of cancer-related death in the Western world with 639,000 deaths worldwide per year (www.who.int/mediacentre/factsheets/fs297/en/). It is postulated that a major cause for these malignancies is a diet rich in fat, refined carbohydrates and animal protein combined with low physical activity (Giovannucci, 2002; Papapolychroniadis, 2004; Satia et al., 2005). Carcinogens like polycyclic aromatic hydrocarbons (PAH) are also present in food. Studies have shown that most food intake of PAHs derives from cereals, oils and fats. Human exposure to PAH occurs by intake of contaminated vegetables and by consumption of broiled food (Larsson et al., 1983). Benzo[a]pyrene (BaP) is a potent and well investigated carcinogen acting as initiator and promotor (Albert

Abbreviations: AhR, aryl hydrocarbon receptor; AhRR, AhR repressor; AIP, aryl hydrocarbon receptor interacting protein; ARE, anti-oxidant response element; ARNT, aryl hydrocarbon receptor nuclear translocator; BaP, benzo[a]pyrene; CYP, cytochrome P450; EROD, ethoxyresorufin *O*-deethylase; GAPDH, glycerolaldehyde-phosphate dehydrogenase; GCLC, glutamate-cysteine ligase catalytic subunit; GSTA1, glutathione S-transferase A1; GSTP1, glutathione S-transferase P1; K, kaempferol; Keap 1, kelch-like ECH-associated protein; Nrf2, nuclear factor erythroid 2-related factor; NQO1, NADPH quinone oxidoreductase 1; PAH, polycyclic aromatic hydrocarbons; PKC, protein kinase C; Q, quercetin; TCCD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element.

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et al., 1991). Hattemer-Frey and Travis (1991) showed that humans are exposed to BaP by air, water and particularly by food which contributes to 97% of the human exposure. It is estimated that the ingested weekly dose of BaP from smoked food varies between 0.01 and 4.0 μg/person (Lioy et al., 1988). It is known that secondary plant compounds such as flavonoids may at least partially counteract the adverse effects of toxicants and procarcinogens. For instance, polyphenolic flavonoids scavenge free oxidative radicals and pose anti-oxidant, anti-thrombotic and anti-carcinogenic activities. Usually, carcinogens and potentially beneficial compounds are concomitantly taken up by food consumption and thus concertedly act on molecular processes in intestinal cells after their mobilization in the gastrointestinal tract. In fact, polyphenolic flavonoids are predominant phytochemicals in our diet and provide much of the flavor and color to fruits and vegetables. The most abundant flavonoids are the flavonols quercetin and kaempferol which exist in plants as a variety of glycosides and in their aglycone form (Lauren et al., 2009). As dietary supplements, flavonoids are available in the aglycone or the glycosylated form, such as quercetin and rutin. Dependent on the structure of the carbohydrate moiety, the glycosides differ in their bioavailability in humans which can be either higher or lower than that of the aglycone (Scalbert and Williamson, 2000; Manach et al., 2005). Furthermore, the bioavailability of flavonoids is influenced by the colonic microflora which is responsible for the deconjugation of the respective glycosides. Studies have shown that quercetin and kaempferol are absorbed by the human gut (Hollman and Katan, 1997; Paganga and Rice-Evans 1997; Petri et al., 2003; Scholz and Williamson, 2007) and also by Caco-2 cells (Tian et al., 2009). Due to these facts, we used the aglycones quercetin and kaempferol for our *in vitro*-studies.

Many carcinogens have to be enzymatically activated to attain their final carcinogenicity and in many cases the primary procarcinogens induce enzymes which are responsible for their own metabolism. This applies also for BaP which is studied here. It is well established that BaP induces cytochromes P450 (CYP) CYP1A1, CYP1A2, CYP1B1 and CYP2S1 via the aryl hydrocarbon receptor (AhR) (Nebert et al., 1993; Tsuchiya et al., 2003b; Rivera et al., 2007). From these enzymes, only those of the CYP1-family are involved in the metabolism of BaP. In addition, enzymes of the CYP3A-subfamily also contribute to BaP metabolism by using primary metabolites formed by CYP1-members as substrates (Shimada et al., 1989). The mentioned enzymes are tissue-specifically expressed with CYP1A2 representing the major constitutively expressed CYP1-enzyme in the liver. In Caco-2 cells, both, CYP1A1 and CYP1B1 have been detected and are inducible by PAH at least at the mRNA level (Lampen et al., 2004). From the various human CYP3A-isoenzymes, CYP3A4 is obviously the major species expressed in Caco-2 cells (Sambuy et al., 2005). Thus, the intestinal cells are capable of forming chemically reactive metabolites from BaP resulting in procarcinogen activation. It has been shown that the intestinum has the capacity to convert nearly 100% of orally administered PAH into metabolites before they enter the circulation (Cavret and Feidt, 2005).

Naturally occurring plant compounds present in the food can interfere with pathways initiated by BaP either as agonists or antagonists and may thus modulate the final outcome of BaP-mediated alterations. To mechanistically understand the possible beneficial effects of plant compounds on BaP-dependent activities, we examined the effects of two flavonoids, quercetin and kaempferol, in the absence or presence of BaP on components and target genes of the AhR-pathway. This selective approach has been chosen to identify potential flavonoid targets within the clearly BaP-modulated AhR pathway. The receptor complex includes several and changing protein components, such as hsp90, AIP and ARNT dependent on its activation state and cellular localization (Fujii-Kuriyama and Kawajiri, 2010). Furthermore, AhR regulated genes can be silenced by a dimer between ARNT and the AhR repressor (AhRR) which blocks respective xenobiotic response elements due to missing transactivation activity (Oshima et al., 2007). The selected flavonoids for this study have a very similar structure and differ only by the number of hydroxyl-groups in ring B, two in quercetin and one in kaempferol. The ortho-orientation of the OH-groups in the B-ring of quercetin allows for redox-reactions as an *o*-quinone can be formed (Rietjens et al., 2005). Indeed, the efficiency of flavonoids to induce antioxidant-responsive element (ARE)-mediated gene expression correlates with their redox properties (Lee-Hilz et al., 2006). Based on these facts and the mechanistic connection of the AhR with the Nrf2-pathway, we further studied the expression of Nrf2, its natural inhibitor protein Keap1 and its target genes glutathion-S-transferases GSTA1 and GSTP1, NADPH quinone oxidoreductase (NQO1) and glutamate-cysteine ligase catalytic subunit (GCLC). The Nrf2 gene includes three AhR-binding xenobiotic response element like (XREL) in its regulatory region (Miao et al., 2005) while Nrf2-binding ARE are present in the 5′-upstream region of its target genes (Joseph et al., 1994; Sakai and Muramatsu, 2007; Vollrath et al., 2006; Wild et al., 1998). This is the molecular basis for the AhR-Nrf2 cross-talk.

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene and dimethyl sulfoxide (DMSO) were purchased from Sigma, Taufkirchen,Germany. Kaempferol and quercetin-dihydrate (Fluka, Switzerland) were dissolved in dimethyl sulfoxide (Sigma, Germany) and stored at −20 °C. QIAzol Lysis Reagent was purchased from Quiagen, Netherlands, neutral red from Biochrom, Germany and T-PER Tissue Protein Extraction Reagent from Pierce, USA. All other standard laboratory chemicals were purchased in p.a. quality from Sigma–Aldrich (Germany), Merck (Germany) and Riedel-de Haën (Germany).

2.2. Cell culture and exposure to xenobiotics

Caco-2 cells (human colorectal adenocarcinoma) were purchased from Cell Lines Service, Eppelheim, Germany (catalogue No. 300,137, passage No. 32). The cells were grown in D-MEM (high glucose: 4.5 g/l, Invitrogen, No. 31053–028) containing 10% FCS, 1% L-glutamine, 1 % non-essential amino acids (NEAA) and 1% penicillin/streptomycin. Medium and supplements were obtained in sterile conditions from Invitrogen GIBCO, UK. FCS was purchased from Biochrom, Germany. For exposure of cells to the xenobiotics, Caco-2 cells were seeded out at $3–4 \times 10^6$ cells/75 cm^2 flask. Forty-eight hours after seeding, the cells were incubated with different concentrations of BaP (0.01–10 μM) or the flavonoids quercetin or kaempferol (0.5–10 μM) for further 48 h. For the co-exposure experiments cells were pre-incubated with each flavonoid (10 μM) for 30 min prior to incubation with BaP (1 or 10 μM). The final DMSO concentration in the culture medium was always 0.1% (v/v).

2.3. Neutral-red cytotoxicity assay

The ability of healthy cells to accumulate neutral-red into their lysosomes was used to estimate a non-cytotoxic concentration of the substances used in the experiments. The neutral-red accumulation assay was performed according to the method of Borenfreund et al. (1988).