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Luteolin enhances the bioavailability of benzo(a)pyrene in human colon carcinoma cells

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ABSTRACT

We investigated the effect of luteolin, a plant-derived flavonoid, on benzo(a)pyrene (B(a)P)-stimulated drug metabolism and transport in human colon carcinoma cells. While luteolin treatment inhibited B(a)P-induced expression and activity of arylhydrocarbon receptor-dependent cytochrome P450 enzymes, the overall activity of UDP-glucuronosyltransferases and sulfotransferases was not affected by luteolin, indicating that luteolin affects phase-I but not phase-II function. Luteolin exposure decreased apical transport of B(a)P metabolites due to its interaction with the transporter breast cancer resistance protein. Inhibitor studies provide a first clue to the mechanism of luteolin-mediated inhibition of this transporter. The inhibition of both phase-I metabolism as well as phase-III transport by luteolin resulted in a 3-fold intracellular accumulation of radioactively labeled B(a)P. Our data reveal that luteolin is able to interfere with crucial steps of drug metabolism and thereby enhances the bioavailability of B(a)P. These findings are of special importance regarding future benefit-risk evaluations of preventive flavonoid usage.

Introduction

Epidemiological studies indicate that fruit- and vegetable-rich diets can reduce the incidence of cardiovascular diseases, diabetes, stroke and cancer [1]. Most of these protective effects are attributed to phenolic secondary plant metabolites, especially the large group of flavonoids that possess strong anti-oxidative properties. Beside these scavenging qualities, flavonoids may alter cellular function through selective actions on different signal transduction pathways responsible for proliferation, apoptosis, cell growth and differentiation [1]. The most intensively investigated flavonoid is quercetin, a flavone that became prominent due to its widespread beneficial effects. Another flavone that exerts significant anti-carcinogenic effects and therefore moves into focus of modern cancer prevention and therapy is luteolin. As reviewed in Refs. [2,3] several studies revealed that luteolin exposure inhibits cancer cell proliferation, tumor growth and metabolic activation of pro-carcinogens. Moreover, luteolin induces the cellular anti-oxidative gene battery, cell cycle arrest and apoptosis in transformed cells. Luteolin can be found in thyme, celery, artichoke, apples and several other plants, and occurs mostly in its glycosylated form. Both forms of luteolin, pure and glycosylated, were identified in human serum [4]. The average daily uptake of luteolin is about 1 mg or less [3]. Due to their presumed beneficial action on human health a large public interest is put on flavonoids leading to the fact that dietary supplements and herbal remedies became another notable source of luteolin exposure.

Since the gut is the first place of contact, flavonoids are thought to act preventive against the development of colorectal cancer diseases that are, at least in part, provoked by cigarette smoke and intake of charcoal-grilled meat that contain carcinogenic compounds like polycyclic aromatic hydrocarbons (PAH)[1] and heterocyclic aromatic amines [5]. Several of these substances require metabolic activation in order to interact with cellular DNA and provoke gene mutations [6]. One class of those catalyzing enzymes are the cytochrome P450 (CYP) family 1 monooxygenases, which have also been linked to the development of different tumor diseases [6]. The expression of CYP1 genes is regulated by the arylhydrocarbon receptor (AhR), a ligand-activated transcription factor that mediates the biochemical and toxic effects of dioxins and related

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[1] *Abbreviations used:* ABC, ATP-binding cassette; AhR, arylhydrocarbon receptor; B(a)A-diOH, benzo(a)anthracene-*trans*-1,2-dihydrodiol; B(a)P, benzo(a)pyrene; BCRP, breast cancer resistance protein; BP3G, B(a)P-3-glucuronide; BP3S, B(a)P-3-sulfate; CYP, cytochrome P450; DMSO, dimethylsulfoxide; EROD, ethoxyresorufin-*O*-deethylase; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; MDR, multidrug resistance; MRP, multidrug resistance protein; 4-MUF, 4-methylumbelliferone; NBD, nucleotide binding domain; PAH, polycyclic aromatic hydrocarbons; SPE, solid phase extraction; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

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environmental pollutants [7]. In absence of a ligand the AhR is trapped in a cytosolic multiprotein complex. Upon ligand binding, this complex dissociates and the AhR shuttles into the nucleus, dimerizes with the AhR nuclear translocator and binds to so called xenobiotic-responsive elements in the promoters of target genes to induce gene expression. The AhR gene battery encompasses several phase-I enzymes but also encodes for a couple of phase-II proteins, for instance some UGT1 enzymes [7]. While UGT2 proteins glucuronidate endogenous substrates like bile acids and steroid hormones, members of the UGT1 family mainly catalyze the glucuronidation of oxidized PAHs and other xenobiotics. UGT-driven glucuronidation has also been shown to be a major metabolic pathway for flavonoids like quercetin, luteolin and galangin [8,9]. The substrate spectrum of UGT enzymes overlaps, at least in part, with target substances of other phase-II enzymes like cytosolic sulfotransferases (SULT). SULTs catalyze the sulfate conjugation of many hormones, neurotransmitters and xenobiotics and therefore play an important role in detoxification. In contrast to that, sulfation is a considerable step in the host activation of pro-carcinogens. Several flavonoids inhibit SULT activity and thereby reduce the extent of pro-carcinogen activation which contributes to the chemopreventive property of these polyphenols [10,11]. Although the functional role of the AhR in regulation of SULT expression is currently under discussion, recent studies in mice indicate that some isoenzymes (e.g., SULT1A1, 5A1) are inducible by classical AhR ligands [12,13].

Recently, it was shown that exposure of Caco-2 cells towards AhR agonists resulted in an enhanced expression of the ATP-binding cassette (ABC) transporter breast cancer resistance protein (BCRP) [14]. In addition, this study revealed that BCRP catalyzes the transport of sulfated and glucuronidated B(a)P metabolites. BCRP is a half-transporter that is expressed in a variety of tissues and organs like colon, small intestine, liver, kidney and placenta [15]. ABC transporter plays an important role especially in tissues that have barrier-functions like the gut or the blood–brain barrier [16]. The high expression rate of ABC transporters in several tumors seems to be associated with multidrug resistance (MDR). The bioavailability of flavonoids depends on transport from intestinal cells back into the lumen by ABC transporter [17].

Taken together the activated AhR is able to influence all three phases of drug metabolism, pointing to the importance of this signaling pathway for normal physiological function. Interestingly, luteolin and several other flavonoids have been reported to inhibit AhR signaling [18–20] and were therefore supposed to act preventive against chemical-associated diseases. Our current study focuses on the influence of luteolin on AhR-regulated xenobiotic metabolism in human colon carcinoma cells. In order to ascertain putative harmful effects of highly concentrated luteolin formulations on human health we performed *in vitro* assays that include analyses of AhR-mediated mRNA expression and enzyme activities, transport assays as well as studies on intracellular B(a)P accumulation. The obtained data reveal that luteolin inhibits B(a)P-stimulated expression and activity of CYP1 enzymes as well as BCRP-mediated transport of B(a)P metabolites resulting in an intracellular accumulation of this carcinogenic PAH.

Materials and methods

All chemicals if not indicated otherwise were obtained from Sigma–Aldrich (Munich, Germany), PBS, cell culture media and supplements from PAA (Coelbe, Germany). The Gold RNA kit was purchased from PEQLAB (Erlangen, Germany). MMLV reverse transcriptase was obtained from Invitrogen (Karlsruhe, Germany). SensiMix PLUS SYBR was delivered by Quantace (London, England). All primers were synthesised by MWG Biotech AG (Ebersberg, Germany). The CBQCA kit was delivered by Molecular Probes (Leiden, The Netherlands). Six-well transwell inserts were retrieved from Corning (Amsterdam, The Netherlands). B(a)P-3-sulfate, B(a)P-3-glucuronide and B(a)A-diOH were purchased by MRI (Kansas City, USA), SPE cartridges were delivered by Malinckrodt-Baker (Deventer, The Netherlands). The Grom-Sil PAH 5 μm column was obtained from Grace Davison Discovery (Lokeren, Belgium) Tritium-labeled B(a)P was acquired from Amersham Biosciences (Braunschweig, Germany). KO143 was delivered by Sigma–Aldrich (Munich, Germany).

Cell culture and treatment

Caco-2 cells (Lot. No. 6) were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany) and cultured in RPMI 1640 medium, supplemented with 10% (v/v) fetal calf serum, 0.75% (v/v) sodium bicarbonate and penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were passaged at 70% confluence and used between passages 8 and 30. Test compounds were dissolved in DMSO and added to culture media at the indicated concentrations. The final DMSO concentration was 0.2% (v/v).

Quantitative real-time PCR

Cells (2×10^5) were seeded into six-well plates and cultured for 10 days. Media were replaced every second day. Cells were treated with either 15 μM or 25 μM luteolin for 1 h and subsequently co-treated with 1 μM B(a)P for 24 h. Cells were washed with PBS and RNA was isolated using the Gold RNA kit. For each sample 0.5 μg of total RNA was reverse transcribed using MMLV reverse transcriptase in a total volume of 20 μl. Three microliters of cDNA of a 1:3 dilution were used for real-time PCR in a Corbett-Rotor Gene 300 light cycler (Qiagen, Hilden, Germany) with SensiMix PLUS SYBR. The oligonucleotides for amplification of UGT1A1 were 5′-CCTTTGCCTCAGAATTCCTTC-3′ (sense) and 5′-ATTGATCCCAAA GAGAAAACCAC-3′ (antisense). Other oligonucleotides were described previously: CYP1A1 [21], β-actin [21], SULT1A1 [22], UGT1A6 [23] and BCRP [14]. Gene expression was normalized to β-actin and plotted as fold of untreated control.

Ethoxyresorufin-O-deethylase (EROD) assay

For ethoxyresorufin-*O*-deethylase (EROD) activity assays, 1×10^5 Caco-2 cells were seeded into 48-well plates and cultured for 10 days. Medium was replaced every second day. Cells were pre-treated for 1 h with 15, 25 and 50 μM luteolin and subsequently exposed to 1 μM B(a)P for 24 h. EROD assays were performed according to Ref. [24]. Cells were incubated with 2.5 μM 7-ethoxyresorufin and EROD activity was monitored in a Fluoroskan Ascent fluorometer (Thermo Fisher, Waltham, USA) at 544 nm excitation and 590 nm emission. Resorufin increase was monitored for 20 min. The amount of formed resorufin was calculated using a standard curve. Following EROD analysis cells were washed with PBS and protein content was determined using the CBQCA kit. Fluorescence was measured at 485 nm excitation and 538 nm emission. Data are shown as pmol/mg protein and minute.

UDP-glucuronosyltransferase (UGT) assay

The UGT assays were done according to Ref. [25]. Cells were cultured as described for the EROD assay. Cells were treated with 50 μM luteolin and 1 μM B(a)P for 24 h. After exposure, cells were washed with PBS and incubated for 1 h with serum free medium containing 100 μM 4-methylumbelliferone (4-MUF). Finally, 10 μl of each sample were added to 190 μl of 10 mM NaOH in a 96-well