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Epigallocatechin-3-gallate does not affect the activity of enzymes involved in metabolic activation and cellular excretion of benzo[a]pyrene in human colon carcinoma cells

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ABSTRACT

Benzo[a]pyrene (B[a]P) and related procarcinogens found in cigarette smoke and roasted foodstuff require metabolic activation to build mutagenic DNA adducts that may cause tumor diseases like colorectal cancer. The major B[a]P-activating enzymes belong to the cytochrome-P450 (CYP)-1 family and are regulated by the aryl hydrocarbon receptor (AhR). Previous studies have indicated that an inhibition of AhR is accompanied with a reduced metabolic activation of B[a]P and therefore may act protective against carcinogenesis. We investigated if the green tea flavonoid (−)-epigallocatechin-3-gallate (EGCG), a known AhR inhibitor, is able to influence B[a]P-metabolizing and B[a]P–transporting enzymes in human Caco-2 colon carcinoma cells. Strikingly, treatment with EGCG did neither affect constitutive and B[a]P-inducible expression of CYP1A1 and UDP-glucuronosyltransferase (UGT)-1A1 nor overall CYP1 and UGT enzyme activities, indicating that EGCG does not antagonize the AhR in Caco-2 cells. Since flavonoids were also identified to enhance the activity of B[a]P-carrying transporter, we analyzed if EGCG exposure alters cellular excretion of B[a]P conjugates. In contrast to the positive control fisetin, EGCG did not affect cellular excretion of B[a]P metabolites. Our data provide evidence that EGCG does not alter the metabolism and transport of B[a]P in Caco-2 cells, and thus may not protect against procarcinogenic food contaminants.

1. Introduction

Due to comprehensive epidemiological studies plant-derived polyphenolic compounds have been associated with a lower risk for several diseases, including diabetes, stroke, cardiovascular diseases and certain types of cancer (Crozier et al., 2009). Especially flavonoids contained in green tea [*Camellia sinensis* L. Ktze.] are commonly regarded as protective agents that contribute to human health (Cabrera et al., 2006). As a result, concentrated formulations of green tea extract have become one of the most popular dietary supplements in industrialized countries. The major active constituent of green tea is (−)-epigallocatechin-3-gallate (EGCG), a compound that was shown to interfere with several transcription factors and growth factors involved in malignant transformation or abnormal cell proliferation, such as NF-κB (Gupta et al., 2004), insulin-like growth factor-1 (Shimizu et al., 2005a), vascular endothelial growth factor (Jung et al., 2001), several members of the epidermal growth factor receptor (EGFR) family (Shimizu et al., 2005b) and others.

Since the gut is the first place of contact, EGCG and related flavonoids are thought to act preventive against the development of nutrition- and lifestyle-related colorectal cancers (Weisburger and Chung, 2002). Typical procarcinogens contained in cigarette smoke, roasted coffee and charcoal-grilled meat are polycyclic aromatic hydrocarbons (PAH) and heterocyclic aromatic amines (Ferguson, 2002; Jagerstad and Skog, 2005). Many of these procarcinogenic compounds require metabolic activation in order to form DNA adducts that may lead to persistent gene mutations and subsequent tumor initiation (Ioannides and Lewis, 2004). The probably best examined enzymes regarding metabolism of PAHs like benzo[a]pyrene (B[a]P) belong to the cytochrome P450 (CYP) subfamily 1 of monooxygenases (Ioannides and Lewis, 2004). The expression of these CYP1 isoforms is under control of the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor that is known to mediate the toxicity of PAHs, dioxins

Abbreviations: AhR, aryl hydrocarbon receptor; 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; EGCG, (−)-epigallocatechin-3-gallate; EROD, ethoxyresorufin-O-deethylase; HSP90, heat-shock protein 90; PAH, polycyclic aromatic hydrocarbons; UGT, UDP-glucuronosyltransferase.

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and related environmental contaminants (Abel and Haarmann-Stemmann, 2010). In absence of a ligand, the AhR is located in the cytosol trapped in a multiprotein complex consisting of a heat-shock protein 90 (HSP90) dimer, Hepatitis Virus X associated protein 2 and the co-chaperone p23. Upon ligand binding this multiprotein complex dissociates and the AhR translocates into the nucleus and dimerizes with the AhR nuclear translocator. The resulting heterodimer then binds to xenobiotic-responsive elements in the promoters of target genes to stimulate their expression. The AhR gene battery encodes for enzymes of xenobiotic metabolism as well as for proteins involved in regulation of cell growth and differentiation. The prototype drug-metabolizing targets of the AhR are CYP1A1, CYP1A2 and CYP1B1, but it also regulates the expression of phase II enzymes like UDP-glucuronosyltransferase (UGT) 1A1 or glutathione S-transferase A2 (Abel and Haarmann-Stemmann, 2010). The importance of a functional AhR cascade for the metabolic activation of B[a]P and related PAHs was proven *in vivo*, by demonstrating that AhR-deficient mice are protected against the carcinogenicity of those compounds (Nakatsuru et al., 2004; Shimizu et al., 2000).

Several plant polyphenols were identified to antagonize the activated AhR and downstream expression and activity of CYP1 enzymes, a mechanism that is thought being protective against PAH-induced DNA-damage (Ciolino et al., 1998; Takemura et al., 2010). In parallel, most of these flavonoids activate the transcription factor NF-E2 p45-regulated factor, the master regulator of the antioxidant response, which regulates (sometimes in concert with the AhR) the expression of several phase II conjugating enzymes that subsequently detoxify reactive phase I metabolites (Surh et al., 2008). Recently, AhR-modulating flavonoids were shown to induce the expression of breast cancer resistance protein (BCRP), an ATP-binding cassette transporter known to excrete B[a]P metabolites (Ebert et al., 2007).

Green tea constituents protect against B[a]P-induced mutations in transgenic mice (Jiang et al., 2001) and Quattrochi and colleagues have shown that green tea catechins can significantly repress dioxin-induced expression of CYP1A1 and CYP1A2 in human hepatoma cells (Williams et al., 2000). Subsequent investigations have identified EGCG as the most potent AhR-antagonizing flavonoid contained in green tea (Palermo et al., 2003). Noteworthy, EGCG does not bind directly to the AhR ligand binding domain but interferes with the HSP90 chaperones to disturb proper AhR signaling (Palermo et al., 2005). Further experiments revealed that EGCG exposure also inhibits the activity of other HSP90 client proteins, for instance HER2, a member of the EGFR family that was already reported to be sensitive towards EGCG treatment in human colon carcinoma cells (Shimizu et al., 2005b; Yin et al., 2009). These findings imply that inhibition of HSP90-dependent signaling molecules is probably a central and relevant mechanism contributing to the chemopreventive properties of EGCG.

Hence, we investigated in this study if EGCG has the potential to modulate AhR-regulated expression and activity of B[a]P-metabolizing enzymes in human Caco-2 colon carcinoma cells. Further on, we asked if EGCG exposure affects the cellular excretion of the major B[a]P phase II metabolites. Both endpoints would shed light on the question if EGCG may be a suitable agent to prevent colon carcinogenesis provoked by food-borne PAHs.

2. Material and methods

2.1. Media and chemicals

Cell culture media and supplements were obtained from PAA (Coelbe, Germany). B[a]P-3-sulfate, B[a]P-3-glucuronide and benzo[a]anthracene-*trans*-1,2-dihydrodiol were purchased by MRI (Kansas city, USA). The MMLV reverse-transcriptase was obtained from Invitrogen (Karlsruhe, Germany) and SensiMix PLUS SYBR from Quantace (London, England). Six-well transwell inserts were delivered by Corning (Amsterdam, The Netherlands) and solid phase extraction cartridges by Mallinckrodt–Baker (Deventer, The Netherlands). The Grom-Sil PAH, 5 μm column was obtained from Grace Davison Discovery (Lokeren, Belgium). The CBQCA kit was supplied by Molecular Probes (Leiden, The Netherlands). Oligonucleotides were synthesised by Eurofins MWG Operon (Ebersberg, Germany). All chemicals (including flavonoids) not contained in this list were obtained from Sigma–Aldrich (Munich, Germany) at highest available purity.

2.2. Cell culture and treatment

The Caco-2 cell line (Lot. No. 6) was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C in RPMI1640 medium plus 10% (v/v) fetal calf serum, 0.75% (v/v) sodium bicarbonate and penicillin/streptomycin mixture. Cells were splitted at 70% confluence and used between passages 8–30. EGCG, fisetin, luteolin, baicalein, B[a]P and B[a]P derivates were dissolved in DMSO and added to culture media at the indicated concentrations. For all co-exposure experiments, cells were pre-treated for 1 h with the respective flavonoid and subsequently co-exposed to 1 μM B[a]P. The final DMSO concentration was 0.2% (v/v). All test compounds were used at non-cytotoxic concentrations as determined by LDH leakage measurement.

2.3. Quantitative real-time PCR

For quantitative mRNA expression analyses 2×10^5 cells were seeded into six-well plates and cultured for ten days. Cells were pre-treated for 1 h with 15 μM, 25 μM and 50 μM EGCG and subsequently co-treated with 1 μM B[a]P or solvent for 24 h. Total RNA was isolated and 0.5 μg of each sample were reverse transcribed using MMLV reverse transcriptase. Transcripts were amplified using SensiMix PLUS SYBR mix in a Corbett-Rotor Gene 300 light cycler (Qiagen, Hilden, Germany). The oligonucleotides for β-actin, CYP1A1 and UGT1A1 were described previously (Bothe et al., 2010). Gene expression was normalized to β-actin and plotted as fold of untreated control.

2.4. Ethoxyresorufin-O-deethylase (EROD) assay

1×10^5 Caco-2 cells were seeded into 48-well plates and cultured for ten days. Cells were pre-treated for 1 h with 15 μM, 25 μM and 50 μM EGCG and subsequently treated for 24 h with 1 μM B[a]P or solvent. Cells were incubated with 2.5 μM 7-ethoxyresorufin and 10 μM dicoumarol in serum-free media 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 (CBQCA). Fluorescence was measured at 485 nm excitation and 538 nm emission. Data are shown as pmol/mg protein and min.

2.5. UDP-glucuronosyltransferase (UGT) assay

The UGT assays were done as described in (Bothe et al., 2010), cells were cultured as described for EROD assay. Caco-2 cells were treated for 24 h with 50 μM EGCG or 1 μM B[a]P. Subsequently, cells were washed with PBS and incubated for 1 h with serum-free medium containing 100 μM 4-methylumbelliferone. 10 μl of