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Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2

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A B S T R A C T

Epigallocatechin gallate (EGCG) has been shown to be protective in various experimental models of liver injury, although opposite effects have also been reported. Since its effect on biliary physiology has not been thoroughly investigated, the present study evaluated effect of EGCG on bile flow and bile acid homeostasis in rats. Compared to controls, EGCG treatment decreased bile flow by 23%. Hepatic paracellular permeability and biliary bile acid excretion were not altered by EGCG administration, but biliary glutathione excretion was reduced by 70%. Accordingly, the main glutathione transporter on the hepatocyte canalicular membrane, multidrug resistance-associated protein 2 (Mrp2), was significantly decreased at the protein level. EGCG administration also doubled plasma bile acid levels compared to controls. While protein levels of the main hepatic bile acid transporters were unchanged, the rate-limiting enzyme in the bile acid synthesis, Cyp7a1, was significantly increased by EGCG. Enhanced bile acid synthesis in these animals was also confirmed by a 2-fold increase in plasma marker 7α-hydroxy-4-cholesten-3-one. In contrast, EGCG markedly downregulated major bile acid transporters (Asbt and Ostα) and regulatory molecules (Shp and Fgf15) in the ileum. When EGCG was coadministered with ethinylestradiol, a potent cholestatic agent, it did not show any additional effect on the induced cholestasis. This study shows ability of EGCG to raise plasma bile acid concentrations, mainly through Cyp7a1 upregulation, and to decrease bile production through reduction in Mrp2-mediated bile acid-independent bile flow. In conclusion, our data demonstrate that under certain conditions EGCG may induce cholestasis.
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1. Introduction

Bile production is one of the essential functions of the liver and is vital for both proper digestion of dietary lipids and elimination of lipid soluble endo- and xenobiotics, including drugs and toxins. The key step in the whole process is secretion of osmotically active compounds, especially bile acids and glutathione, across the hepatocyte canalicular membrane into the bile capillaries, followed by passive movement of water (Esteller, 2008). To generate the driving force for bile flow, solutes are transported into bile against a concentration gradient by energy-dependent transporters; bile salt exporting pump (Bsep) is essential for bile acid-dependent bile flow and multidrug resistance-associated protein (Mrp) 2 is crucial for bile acid-independent bile flow, which is based on the transport of glutathione and glutathione conjugates. Nevertheless, since bile

Abbreviations: Asbt, apical sodium-dependent bile salt transporter; ALT, alanine transaminase; AST, aspartate transaminase; Bsep, bile salt exporting pump; CDCA, chenodeoxycholic acid; Cyp7a1, cholesterol 7α-hydroxylase; C4, 7α-hydroxy-4-cholesten-3-one; EE, ethinylestradiol; EGCG, epigallocatechin gallate; Fgf, fibroblast growth factor; Fxr, farnesoid X receptor; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; Mrp, multidrug resistance-associated protein; Ntcp, Na^+-taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; Ost, organic solute transporter; Shp, small heterodimer partner.

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acids are synthesized from cholesterol and the majority of their pool undergo excessive enterohepatic recirculation, several other transporters and enzymes in the liver and intestine are also crucial for proper functionality of the whole process (Esteller, 2008).

Epigallocatechin gallate (EGCG), the main active component of green tea, is currently a widely used natural compound available in numerous products either in purified form or as a part of tea or tea extracts. EGCG has demonstrated a great variety of health benefits, which are mainly related to its potent anti-oxidant, anti-inflammatory and anticancer activities (Singh et al., 2011). Among these, one of the prominent effects is its hepatoprotective action in various forms of liver injury (Ramesh et al., 2009; Ren et al., 2011; Tipoe et al., 2010), including cholestatic injury induced by bile duct ligation (Kobayashi et al., 2010; Zhong et al., 2003). Although most of these effects are ascribed to the anti-inflammatory activity of the compound, Li et al. (2012) has recently reported a unique feature of EGCG to stimulate farnesoid X receptor (Fxr), the master regulator of bile acid homeostasis, selectively in the intestine and thus increase expression of its target gene fibroblast growth factor 15 (Fgf15, a mouse homologue of human FGF19). This mechanism has been suggested to protect the liver against cholestasis as it may significantly reduce bile acid synthesis in the liver (Modica et al., 2012). However, the effect of EGCG on bile flow and corresponding mechanisms has not yet been thoroughly studied. In contrast, our recent data showed a bile flow decreased in EGCG-exposed rats (Hirsova et al., 2012). In fact, such information is of great interest since several cases of cholestasis have been reported in humans after either administration of high dose of EGCG/green tea or coadministration with other potentially cholestatic agents, such as ethinylestradiol (Mazzanti et al., 2009), a widely used contraceptive. The mechanism involved may therefore account for possible toxic effects of EGCG reported recently (for review see Stickel et al., 2011).

The aim of the present study was to identify the effect of EGCG on overall bile production as well as to analyze separate effects on bile acid-dependent and bile acid-independent bile flow. In view of the current concept that FXR activators may be useful in treating intrahepatic cholestasis due to suppression of bile acid synthesis and promotion of bile acid efflux from hepatocytes (Zhu et al., 2011), the effect of EGCG in our study was further analyzed in a model of intrahepatic cholestasis induced by ethinylestradiol (EE).

2. Materials and methods

2.1. Materials

Epigallocatechin gallate and ethinylestradiol were obtained from Cayman Chemical and Sigma–Aldrich, respectively. Rhamnose and melibiose were purchased from Sigma.

2.2. Animals and experimental design

All animal experimental protocols were approved by the Animal Research Committee of the Faculty of Medicine in Hradec Kralove, Charles University in Prague, Czech Republic. Female Wistar rats (230–250 g) were obtained from Anlab (Czech Republic) and were housed under controlled temperature and light conditions, on a natural 12 h light–dark cycle. Animals had free access to water and food throughout the experiment, but were fasted overnight before sacrifice. Because the decrease in bile flow by EGCG was first noticed in our previous study (Hirsova et al., 2012), we decided to use the same study design. Briefly, rats were randomly divided into four groups and were administered either EGCG 50 mg/kg body weight (EGCG group) or saline (control group, Ctrl) by i.p. injection once daily for 8 consecutive days. Groups EE and EE-EGCG received also ethinylestradiol (5 mg/kg body weight) s.c. once daily for 5 days, starting on day 4 of saline or EGCG treatment. This dose of EGCG (50 mg/kg) may result in a plasma concentration of 1 μM after 1 h and peak plasma concentration of 2 μM after 2 h. In humans, the plasma concentration of ~1 μM is achievable after either drinking ~8 cups of green tea or ingestion of EGCG-rich extracts (Hirsova et al., 2012).

Bile collection and sacrifice were performed on day 9, i.e. the next day after the last dose of EGCG. Bile collection was carried out as previously described (Hirsova et al., 2012). Plasma and bile samples from the initial collecting period (0–30 min) were used for further analyses of bile acids and glutathione as the early period best reflects the situation under cholestasis.

2.3. Routine plasma biochemistry, bile acid and 7α-hydroxy-4-cholesten-3-one (C4) measurements

Plasma bilirubin levels and liver enzyme activities were determined by routine laboratory methods on Cobas Integra 800 (Roche Diagnostics). Bile acids in plasma and bile were assayed using a commercial kit (Diazyme). Plasma concentrations of C4 were measured by high-performance liquid chromatography (HPLC) as previously described by Lenicek et al. (2008). The chromatographic parameters were: Tessek SGX C18 column (4 mm × 250 mm, 4 μm), acetonitrile:water (95:5, vol/vol) mobile phase, flow rate 1 mL/min, detection/reference wavelength 241/360 nm. The detection limit was 1.2 μg/L.

2.4. Glutathione measurements

Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were analyzed separately using an HPLC method (Kand'ar et al., 2007) on a Shimadzu system with fluorescence detection. The chromatographic conditions employed in the study were as follows: stationary phase SUPELCO Discovery C18 (4 mm × 150 mm, 5 μm), mobile phase methanol:phosphate buffer (15:85, vol/vol, 25 mM, pH 6.0), flow rate 0.5 mL/min, detector set at 350 nm and 450 nm (excitation and emission wavelengths, respectively). The limit of detection was 14 fmol and 5.6 fmol for GSH and GSSG, respectively. Total glutathione was calculated as the sum of reduced and oxidized forms of glutathione.

2.5. Evaluation of paracellular permeability in the liver

We assessed hepatic tight junctional permeability by a dual-sugar test based on quantifying biliary excretion of rhamnose and melibiose (Tomsik et al., 2008). Biliary concentrations of melibiose and rhamnose were determined by HPLC method with a fluorescent detection as previously described (Tomsik et al., 2008). The ratio of biliary excretion of melibiose (disaccharide, which penetrates selectively across the paracellular junctions) to biliary excretion of rhamnose (monosaccharide, which permeates barriers by transcellular diffusion) was used as a marker of the blood–biliary barrier function.

2.6. Rat hepatocyte isolation, culture and treatment

Rat hepatocytes were isolated by collagenase perfusion as previously described (Kand'ar et al., 2007) and cultured in William's E medium supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 IU/mL), streptomycin (10 mg/mL), dexamethasone (0.1 μg/mL), insulin (0.08 IU/mL) and glucagon (8 ng/mL) in a collagen sandwich configuration. After 24 h, medium was removed and replaced with a fresh serum-free medium containing either DMSO or EGCG at concentrations of 1–20 μmol/L. After 24 h incubation, cells were collected in TRI Reagent (Sigma–Aldrich) for gene expression analysis.

2.7. Quantitative real time RT-PCR

Total RNA from rat liver and ileum were isolated with RNeasy Mini Kit (Qiagen). Total RNA from primary rat hepatocytes was isolated using TRI Reagent (Sigma–Aldrich) and cleaned up with RNeasy MinElute Cleanup Kit (Qiagen). Gene expression was examined by quantitative real-time RT-PCR as previously described (Hirsova et al., 2012). TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits (Supplemental Table A) were purchased from Applied Biosystems. The glyceraldehyde 3-phosphate dehydrogenase gene was used as a reference for normalizing data.

2.8. Western blot

Protein expression in the liver was examined by Western blot as previously described (Hirsova et al., 2012). Briefly, proteins (100 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with appropriate antibodies (Supplemental Table B). Horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare and enhanced chemiluminescence reagents were from Thermo Pierce. Densitometry was performed using ScanMaker i900 (UMAX) and QuantityOne imaging software (BioRad). Protein levels were normalized to beta-actin levels.

2.9. Gene reporter assay

HepG2 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK) and was used within 25 passages after delivery. For transient transfection gene-reporter experiments, HepG2 cells were maintained in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% nonessential amino acids. HepG2 cells were co-transfected

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