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Endoplasmic reticulum stress underlying the pro-apoptotic effect of epigallocatechin gallate in mouse hepatoma cells

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ABSTRACT

It has been recently reported that tea flavanols, including epigallocatechin gallate (EGCG), efficiently inhibit glucosidase II in liver microsomes. Since glucosidase II plays a central role in glycoprotein processing and quality control in the endoplasmic reticulum we investigated the possible contribution of endoplasmic reticulum stress and unfolded protein response (UPR) to the pro-apoptotic activity of EGCG in mouse hepatoma cells. The enzyme activity measurements using 4-methylumbelliferyl-α-D-glucopyranoside substrate confirmed the inhibition of glucosidase II in intact and alamethicin-permeabilized cells. EGCG treatment caused a progressive elevation of apoptotic activity as assessed by annexin staining. The induction of CHOP/GADD153, the cleavage of procaspase-12 and the increasing phosphorylation of eIF2α were revealed in these cells by Western blot analysis while the induction of endoplasmic reticulum chaperones and foldases was not observed. Time- and concentration-dependent depletion of the endoplasmic reticulum calcium stores was also demonstrated in the EGCG-treated cells by single-cell fluorescent detection. The massive alterations in the endoplasmic reticulum morphology revealed by fluorescent microscopy further supported the development of UPR. Collectively, our results indicate that EGCG interferes with protein processing in the endoplasmic reticulum presumably due to inhibition of glucosidase II and that the stress induces an incomplete unfolded protein response with dominantly pro-apoptotic components.

1. Introduction

Epigallocatechin gallate (EGCG) is the major tea flavanol having a variety of beneficial health-effects. Besides its excellent antioxidant properties, EGCG was repeatedly shown to have chemopreventive and anticarcinogenic actions (Beltz et al., 2006; Shankar et al., 2007). Despite intensive investigation, the molecular targets and mechanisms of action underlying the anticancer effects of EGCG have not been fully elucidated. Several proteins have been reported to interact directly with EGCG, and it became evident that the anti-tumor effects are partly due to pro-apoptotic stimuli. Although the strong antioxidant power makes the polyphenols capable of antagonizing the oxidative-stress-mediated (e.g. hypoxia-induced) apoptosis (Park et al., 2006), EGCG is able to reduce cell viability and initiate apoptosis in various types of tumors (including hepatoma) both in vitro and in vivo (Manson et al., 2007; Moiseeva et al., 2007; Nishikawa et al., 2006; Shimizu et al., 2005).

Our recent observation that tea flavanols inhibit glucosidase II in rat liver microsomes (Gamberucci et al., 2006) raises the possibility that endoplasmic reticulum (ER) stress might be involved in the EGCG-dependent initiation of cell death. Glucosidase II plays a central role in the processing of nascent glycoproteins in the ER lumen by removing certain glucose units from the core oligosaccharide moiety (Roth et al., 2003). One of these glucose units serves as a label of immaturity for the protein quality control system; it is recognized by calnexin and calreticulin, lectin chaperones, which retain the protein in the ER lumen until it reaches the native conformation (Ellgaard and Helenius, 2003; Moremen and Molinari, 2006). The glycoproteins trimmed by glucosidase II can be judged mature and exported towards the Golgi apparatus. However, if they are still misfolded they can be sentenced either to further folding or to degradation, by reglucosylation or demannosylation, respec-

Abbreviations: ATF6, activating transcription factor 6; BiP/GRP78, immunoglobulin heavy chain-binding protein or 78 kDa glucose-regulated protein; CHOP/GADD153, C/EBP homologous protein or growth arrest- and DNA damage-inducible gene 153; eIF2α, eukaryotic translation initiation factor 2, α subunit; EGCG, epigallocatechin gallate; ER, endoplasmic reticulum; ERp72, ER protein of 72 kDa molecular weight; GRP94, 94 kDa glucose-regulated protein; IRE1, inositol-requiring enzyme 1; MUG, 4-methylumbelliferyl-α-D-glucopyranoside; PDI, protein disulfide isomerase; PERK, PKR-like ER protein kinase; UPR, unfolded protein response.

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tively (Ellgaard and Helenius, 2003; Moremen and Molinari, 2006). Disturbance of the protein processing mechanisms in the ER by inhibiting glucosidase II can be expected to cause the accumulation of immature polypeptides, which in turn may lead to the unfolded protein response (UPR).

UPR is a coordinated adaptive mechanism in ER stress, which attempts to maintain or restore the balance between the load of newly synthesized proteins and the folding capacity of the ER (Schroder and Kaufman, 2005). The load is reduced by attenuation of general translation (phosphorylation of eIF2α), and upregulation of ER-associated degradation; while the capacity is increased by induction of ER resident chaperone (GRP94, GRP78/BiP) and foldase (ERp72, PDI) proteins. In addition to these pro-survival efforts, UPR also involves some pro-apoptotic components to eliminate the unhealthy cells in case of prolonged ER stress (Ferri and Kroemer, 2001). Induction of CHOP/GADD153 ("C/EBP homologous protein"/"growth-arrest- and DNA damage-inducible gene 153") leads to a decreased expression of Bcl-2, thus potentiating the intrinsic death pathway. In addition, the sustained release of calcium from the mitochondrial and ER stores (Hetz and Glimcher, 2008) results in the calpain-mediated cleavage of procaspase-12 (or procaspase-4 in humans) (Ma and Hendershot, 2004) and the consequent activation of the caspase cascade.

The inhibition of glucosidase II by EGCG is likely to cause ER stress due to the disturbance of protein maturation and quality control. Therefore, we hypothesized that the activation of the ER-dependent suicidal mechanisms may contribute to the well documented pro-apoptotic anti-tumor action of this flavanol. In the present study, we investigated the effect of EGCG on glucosidase II activity and on apoptosis as well as on the main components of ER stress response in Hepa 1c1c7 mouse hepatoma cells.

2. Materials and methods

2.1. Materials

4-Methylumbelliferyl-α-D-glucopyranoside (MUG), EGCG, alamethicin, thapsigargin, sulfinpyrazone, methanol, trifluoroacetic acid, cell culture medium and supplements were purchased from Sigma Chemical Co. BODIPY® FL thapsigargin and Fluo-3 acetoxymethyl ester were bought from Molecular Probes, Invitrogen. All other reagents were of analytical grade.

2.2. Antibodies

Anti-caspase-12 primary antibody was purchased from Sigma, anti-ERp72 primary antibody from Calbiochem, anti-calnexin primary antibody from BD Biosciences and anti-eIF2α primary antibodies from Cell Signaling Technology. All other primary antibodies were from Santa Cruz Biotechnology. Secondary antibodies were either purchased from Santa Cruz Biotechnology (anti-goat, anti-mouse and anti-rabbit IgGs) or from Sigma (anti-rat IgG).

2.3. Cell culture maintenance and treatment

Mouse hepatoma Hepa 1c1c7 cells (kindly provided by O. Hankinson, University of California at Los Angeles) were grown as monolayers at 37° in 95% air and 5% CO_2 in α-MEM supplemented with 10% fetal bovine serum, glutamine and penicillin–streptomycin. EGCG was dissolved in culture medium and added to the cell culture at 70–80% confluence with 100-fold dilution. The control cells were treated in the same way with EGCG-free medium.

2.4. Glucosidase activity measurements

α-Glucosidase activity of Hepa 1c1c7 cells was determined as the rate of 4-methylumbelliferone production by enzymatic hydrolysis of MUG. Confluent cells were trypsinized, counted, diluted to about 10^7 cells/ml and divided into two aliquots. The cellular membranes were permeabilized (Revesz et al., 2007) in one aliquot by incubation with the pore-forming antibiotic alamethicin (Woolley and Wallace, 1992) (0.2 mg/ml) at 37 °C for 5 min. The cells were then centrifuged (250 × *g* for 4 min) washed with PBS. Finally, the cells were resuspended in serum-free α-MEM at 5×10^6 cells/ml and pre-incubated at 37 °C in the presence or absence of 100 μM EGCG for 0–12 h. The reaction was started by the addition MUG at 50 μM final concentration. Samples were taken after 0, 15, 30, and 45 min, mixed with two volumes of ice-cold methanol and stored at −20 °C until analysis. They were centrifuged (10 min, 4 °C, at 20,000 × *g*) and the 4-methylumbelliferone content of protein-free supernatants was measured by HPLC (Revesz et al., 2007). The enzyme activity was calculated from the rate of the linear increase in methylumbelliferone concentration and expressed as pmol product (methylumbelliferone) produced in 1 min by 10^6 cells.

2.5. Fluorescence microscopy with endoplasmic reticulum labeling

The morphology of the ER in the hepatoma cell cultures was studied using a Nikon Eclipse TE300 microscope equipped with Nikon S Fluor 40× or Planapo 100× objectives and a FITC filter set and Photometrics Cool SNAP HQ CCD camera. Equipments were driven by MetaMorph, Meta Imaging Series™ software. The fluorescent images were acquired after labeling the ER with 0.1 μM BODIPY® FL thapsigargin (Ong et al., 2007). Thapsigargin specifically binds to the sarco-endoplasmic reticulum calcium ATPase and inhibits the calcium pumping. Therefore, the pictures were taken within 2 min to avoid the morphological changes potentially caused by the calcium depleting effect of the dye.

2.6. Measurement of cytosolic free calcium levels

Alterations in the cytosolic free calcium concentration were detected with microscopy using a fluorescent probe. Control and EGCG-treated hepatoma cells were loaded with 3 μM Fluo-3 acetoxymethyl ester for 30 min at 37 °C as detailed earlier (Gamberucci et al., 2004). After loading, cells were kept at 25 °C in Krebs buffer (140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM Hepes pH 7.4). In order to prevent leakage of intracellular Fluo-3, 100 μM sulfinpyrazone was included in the medium (Di Virgilio et al., 1988). The results were not affected by sulfinpyrazone. Thapsigargin (1 μM) or EGCG (10–100 μM) was added to the cells and fluorescence microscopy was carried out using the equipment described above driven by MetaFluor, Meta Imaging Series™ software. At the end of each incubation, ionomycin (10 μM) plus $CaCl_2$ (10 mM) followed by addition of EGTA (20 mM) were added to determine the maximal and minimal fluorescence values, respectively.

2.7. Western blot and apoptosis assays

The protein concentration of the whole cell lysates was measured using the BioRad "micro protein assay kit". Equal amounts of proteins were separated in 9% SDS-PAGE and transferred to PVDF filter membranes by electroblotting. The filter membranes were incubated overnight with the primary antibodies, and for 1 h with the species-specific peroxidase-conjugated secondary antibodies. The antibodies were detected using a chemiluminescence reagent