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PROTECTIVE EFFECT OF QUERCETIN, EGCG, CATECHIN AND BETAINE AGAINST OXIDATIVE STRESS INDUCED BY ETHANOL *IN VITRO*

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Abstract

There is a need for a nontoxic antioxidant agent to be identified which will prevent alcoholic liver disease (ALD) in alcoholic patients. We tested 4 candidate agents: quercetin, EGCG, catechin and betaine, all of which occur naturally in food. HepG2 cells over expressing CYP2E1 were subjected to arachidonic acid, iron and 100 mM ethanol with or without the antioxidant agent. All the agents prevented oxidative stress and MDA/4HNE formation induced by ethanol, except for EGCG. Catechin prevented CYP2E1 induction by ethanol. All the agents tended to down regulate the ethanol-induced increased expression of glutathione peroxidase 4 (GPX4). All the agents, except catechin, tended to reduce the expression of SOD2 induced by ethanol. Heat shock protein 70 was up regulated by ethanol alone and betaine tended to prevent this. All 4 agents down regulated the expression of Gadd45b in the presence of ethanol, which could explain the mechanism of DNA demethylation associated with the up regulation of the gene expression observed in experimental ALD. In conclusion, the *in vitro* model of oxidative stress induced by ethanol provided evidence that all 4 agents tested prevented some aspect of liver cell injury caused by ethanol.

Keywords

Catechin; EGCG; Quercetin; Betaine; Oxidative Stress

INTRODUCTION

There is a need to identify inexpensive natural agents with antioxidant properties, which would prevent alcoholic liver disease (ALD) when consumed just before each drink of an alcoholic beverage. When ALD develops, it is too late to reverse the liver damage beyond what can be accomplished by abstinence. The antioxidant agent needs to be taken just before each alcoholic drink because of the short half life of the antioxidant agents (Lee et al., 2002). To identify the antioxidant properties of 4 candidate agents, catechin, epigallocatechin-3-gallate (EGCG), betaine and quercetin, studies were done in which these 4 agents were tested *in vitro* using ethanol with the synergistic toxicity of iron and arachidonic acid in

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HepG2 cells over expressing CYP2E1, the model of Caro and Cederbaum (Caro and Cederbaum, 2001).

Quercetin has been shown to protect hepatocytes from ethanol-induced oxidative stress by inducing heme oxygenase-1 and it also prevents ethanol-induced oxidative damage in mice and rats (Liu et al., 2010; Molina et al., 2003; Yao et al., 2009; Yao et al., 2007). The low toxicity and pharmacokinetics of quercetin have been established (Ferry et al., 1996; Harwood et al., 2007). However, after oral administration, about 93.3% of quercetin was metabolized in the gut, with only 3.1% metabolized in the liver. Only 5.3% of unchanged quercetin was bioavailable, although the total quercetin absorbed was as high as 59.1% (Chen et al., 2005). Quercetin up regulates the antioxidant peroxiredoxins through the activation of the Nrf2/NRF1 transcription pathway, and protects against oxidative stress (Miyamoto et al., 2010). Another group showed that quercetin protected against liver injury induced by the alcohol in rats (Vidhya and Indira, 2009).

The green tea polyphenol epigallocatechin-3-gallate (EGCG) protects HepG2 cells from CYP2E1-dependent and independent toxicity *in vitro*. Murakami et al showed that EGCG, like epicatechin 3-O-gallate and epicatechin, had an antioxidation activity and suppressed lipid peroxidation in HepG2 cells, in a CYP2E1-independent mechanism (Murakami et al., 2002b). However, the efficiency of EGCG is higher than the 3 other tea catechins (Murakami et al., 2002a). Muto, in 2001, showed that tea catechin, especially EGCG, decreased the activity of CYP2E1, which can explain the antioxidation preventive effect (Muto et al., 2001). In rats fed ethanol and EGCG, fatty livers were reduced, not by reducing CYP2E1 levels, but by increasing fatty acid oxidation (Yun et al., 2007). EGCG decreased free radical adduct formation including 4HNE adduct formation and hepatocyte necrosis, but had no effect on fatty liver and inflammation (Arteel et al., 2002; Zhong et al., 2004). (+)-Catechin is the most powerful free radical scavenger among the members of the different classes of flavonoids. It has the ability to quench singlet oxygen (Tournaire et al., 1993).

One of the first reports made about the protective effect of betaine against liver injury was published in 1964 by a French laboratory (Savel and Leroi, 1964). Twenty years later, a second publication described the potential beneficial effects of betaine preventing liver injury (Barak et al., 1985). The benefits of betaine were also substantiated by Kharbanda (Kharbanda, 2009; Kharbanda et al., 2005) (Kharbanda, 2009) (Kharbanda et al., 2009). Betaine supplements prevented the ethanol-induced increased levels of liver MDA, the decreased levels of GSH, the increased levels of CYP2E1 and the increased ALT levels (Kim et al., 2008). Betaine attenuated fatty liver *in vitro* induced by chronic ethanol feeding in rats (Kharbanda et al., 2005) and *in vivo* when betaine (0.5%) was fed in the diet with ethanol (Barak et al., 1996).

METHODS

Cell Culture

The *in vitro* model used was that of Cederbaum's CYP2E1 over expressing HepG2 cell model, a human hepatoblastoma cell line (Caro and Cederbaum, 2001). The liver cells were transfected to over express CYP2E1 (E47 cell line). The control HepG2 cells were transfected with the mock vector (C34 cell line). We have used this model previously to study oxidative stress induced by ethanol *in vitro* (Bardag-Gorce et al., 2006a; Bardag-Gorce et al., 2006b). This model utilizes Fe⁺⁺-nitrotriactic acid (Fe-NTA) (10 µM) and arachidonic acid (AA) (20 µM) to increase the oxidative stress introduced by the CYP2E1 over expressing HepG2 cells (E47 cells) *in vitro*.

The cell lines were grown in DMEM containing 10% fetal bovine serum (FBS) and 0.5 mg/ml G418 in a humidified atmosphere of 5% CO₂ at 37°C. The cells were subcultured at a 1:10 ratio each week. 20 µM of arachidonic acid was added to the cell media and the cells were incubated at 37°C for 16 h. The media was removed and the cells were washed once with PBS to remove unincorporated arachidonic acid. Fresh media was then added. 25 µM Fe-NTA was added and the cells were incubated for 5 h. The iron-NTA complex (1:3 Fe-NTA) was prepared as described by Cederbaum (Caro and Cederbaum, 2001). After this incubation, the media was replaced with fresh media.

Treatment groups were as follows: 1) ethanol (100 mM), 2) ethanol plus antioxidant agent, 3) antioxidant agent alone, and 4) saline control for each treatment group. Four antioxidant agents were studied as follows: quercetin 25 µg/ml, EGCG 100 µM, catechin 50 µM and betaine 2 mM (final concentrations) (table 1). The cells were incubated for 24 h in sealed 6 well, 100 mm plates for each of the 4 treatments of both E47 and C34 cells. All treatments of the cultures were done in triplicate or quadruplicates. After the treatment the cells were washed with PBS, re suspended in lysis buffer and stored at -80°C.

Quantitative Real-time RT-PCR Assay

Total liver RNAs were extracted with Trizol Plus RNA Purification kit (Invitrogen, Carlsbad, CA). Synthesis of cDNAs was performed with 5 µg total RNA, and 50 ng random hexamer primers using SuperScriptIII RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA). In addition, RT2 Profiler PCR array: Human oxidative Stress and Antioxidant Defense (84 genes) was performed by SA Biosciences (A Qiagen Company, Frederick, MD).

Western Blot Analysis

Proteins (50 µg) from cell lysates were separated by SDS-PAGE gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 h in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol. The membranes were incubated with Nrf2 antibody over night at 4°C (Abcam, Cambridge, MA). Appropriate species polyclonal and monoclonal HRP-conjugated antibodies were used as the secondary antibodies. The membranes were subjected to chemiluminescence detection using luminal, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

Carbonyl Proteins Measurement

Carbonyl was measured using the Oxyblot oxidation detection kit (Millipore, Temecula, CA). The carbonyl groups produced by oxidation were detected by derivitization to 2,4-dinitrophenylhydrazones by reacting with 2,4-dinitrophenylhydrazine (Levine et al., 1994). The dinitrophenyl-derivatized proteins were detected by Western blot analysis using an antibody against derivitized proteins.

Malondialdehyde (MDA) and 4-hydroxynonenal (4HNE) concentration measurement

Malondialdehyde and 4-HNE concentrations were measured in the cytosolic fractions of treated cells using a lipid peroxidation kit (Oxford Biomedical Research Inc., Oxford, MI). This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1) with malondialdehyde (MDA) and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-HNE reacts with two molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm. MDA and 4-HNE concentrations were assessed at the same time in the samples.

Statistics analysis

Data is obtained from 3 to 4 experiments for each cell treatment. The p values were determined by one way analysis of variance followed by all pairwise multiple comparisons (Student-Newman-Keuls Method, using Sigma-Stat software, San Francisco, CA).

Results

E47 cells treated with ethanol showed an increase in CYP2E1 levels when compared to the controls (C34) (Fig 1A). The CYP2E1 over expression was prevented by catechin only. Quercetin and EGCG didn't show any significant effect on CYP2E1 expression. Betaine increased further the expression of CYP2E1 done by ethanol. Lipid peroxidation end products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were also measured. In figure 1B, we found that quercetin, catechin and betaine in combination with ethanol treatment significantly decreased the levels of MDA and 4-HNE in the E47 cells in presence of 100 mM of ethanol (Fig 1B). EGCG did not prevent the ethanol effect and even a further induced increase in MDA and 4HNE concentraformation. In addition, when carbonyl protein levels were measured ethanol induced an increase in carbonyl protein, which was prevented by quercetin and betaine. However, catechin supplementation did not prevent carbonyl protein formation (Figure 1C).

To further analyze the effects of EGCG, two oxidative defense enzyme, NOS2 inducible (Fig 2A) and serum/glucocorticoid regulated kinase 2 were studied (Fig 2B). The PCR array analysis showed that the EGCG decreased the expression of NOS2 in E47 cell lines.

Reactive oxygen species (ROS) are known mediators of intracellular signal cascades. Excessive production of ROS may lead to oxidative stress, loss of cell function, and cell death by apoptosis or necrosis. Lipid hydroperoxides are one type of ROS. Their biological function has not yet been clarified. Phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) is a unique antioxidant enzyme that can directly reduce phospholipid hydroperoxide in mammalian cells (Liang et al., 2007). Quantitative RT-PCR profiles of the effects of the 4 antioxidant agents on the oxidative stress related enzymes were made for comparison purposes. For instance all 4 antioxidant agents tested, down regulated the ethanol induced increased expression of glutathione peroxidase 4 in the E47 cells (Fig 3A). In the case of SOD2, all of agents except catechin tended to prevent the ethanol-induced increased expression in the E47 cells except catechin (Fig 3B). Gadd45b expression was not changed by ethanol alone but tended to be down regulated by all the antioxidant agents on the E47 cells (Fig 3C). Gadd45b acts by unknown ways to demethylate DNA. Therefore, it has a gene up regulation global epigenetic effect (Khavari et al., 2010). This could account for the global up regulation of genes seen in acute and chronic ethanol fed rats (Bardag-Gorce et al., 2007; Bardag-Gorce et al., 2009; Li et al., 2009) which is inhibited by betaine (Li et al., 2010). SESL1 expression was up regulated by quercetin plus ethanol and catechin plus ethanol. It was down regulated by betaine plus ethanol (E47 catechin+ETOH vs E47 ETOH= $p<0.05$) (Fig 3D). Heat shock protein 70 expression was up regulated by ethanol alone in the E47 cells and this tended to be prevented by betaine in ethanol treated E47 cells (Fig 3E). Heat shock protein expression is inhibited by quercetin and acts as a chaperone involved in protein folding, viral synthesis, protein degradation and reactivation of damaged proteins (Gonzalez et al., 2009).

A major mechanism in the cellular defense against oxidative or electrophilic stress is activation of the Nrf2-antioxidant response element in the signaling pathway, which controls the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant capacity (Dong et al.). In our experiment, Nrf2 expression

was not changed by ethanol treatment, but quercetin co treatment increased the expression of Nrf2 by 2 fold.(Fig 4). Quercetin prevented the increase of CYP2E1 and MDA+4HNE levels in the cells, that could be explained by Nrf2 increase (Fig 1A,B).

Discussion

The purpose of this study was to identify the most effective agent to protect liver cells from oxidative stress, in order to use the agent in a clinical trial to prevent alcoholic patients from developing ALD. Each agent was able to prevent oxidative stress changes in some instances. Only, catechin prevented CYP2E1 induction by ethanol, in the E47 cells, a master key in alcohol metabolism and the production of oxidative stress (Cederbaum; Palakodety et al., 1988). Lipid peroxidation leads to formation of cytotoxic agents such as 4-hydroxy-2-trans-nonenal (4-HNE) (Comporti et al.). All the agents tested, except for EGCG, prevented MDA +4HNE accumulation. Probably the most significant result was that all of the agents were affective in preventing oxidative stress except EGCG. The concentration of the agents studied did not affect cell numbers except for quercetin. EGCG increased the lipid peroxidation induced by ethanol. Others have reported that EGCG inhibited MDA and 4HNE formation by ethanol *in vivo* (Arteel et al., 2002). We used a concentration of 100 μ M of EGCG. Cederbaum used 50 μ M EGCG and this concentration prevented ethanol-induced MDA formation (Jimenez-Lopez and Cederbaum, 2005). It has been reported that EGCG can cause liver damage by inducing oxidative stress (Mazzanti et al., 2009). EGCG may prevent oxidative stress induced liver injury by mechanisms other than preventing lipid peroxidation. For instance, EGCG prevented the reduction of glutathione induced by ethanol *in vitro* (Jimenez-Lopez and Cederbaum, 2005). EGCG also prevented the ethanol-induced decrease in mitochondrial membrane potential *in vitro* (Jimenez-Lopez and Cederbaum, 2005). EGCG *in vivo* reduces fatty liver by enhancing fatty acid oxidation by increasing the pACC and CPT-1 levels (Yun et al., 2007). It also decreases CCl₄-induced liver inflammation, oxidative stress and fibrosis in mice (Tipoe et al., 2010). EGCG prevented the up regulation of NOS2-inducible and SGRK2 expression induced by ethanol in the E47 cells, in the current study. EGCG, quercetin, catechin and betaine decreased glutathione peroxidase 4 in the ethanol treated E47 cells. In the case of SOD2 expression, EGCG, quercetin and betaine tended to prevent the up regulation of the expression of SOD2 caused by ethanol in the E47 cells. The expression of Gadd45b was down regulated by EGCG, quercetin, catechin and betaine compared to ethanol alone in the E47 cells. The expression of SESL1 was up regulated by quercetin and catechin alone and its expression was only diminished by betaine. Betaine also decreased the expression of HSP70.

Recently, Nrf2 has been demonstrated to be a key transcription factor that regulates the induction of antioxidant genes (Nguyen et al., 2009; Zhang, 2006). Under basal conditions, Nrf2 is mainly in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (Keap1), which in turn facilitates the ubiquitylation and subsequent proteolysis of Nrf2 in a constitutive manner (McMahon et al., 2003). In response to oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus and elicits the antioxidant response by induction of a battery of gene products, including antioxidant and phase II detoxification enzymes (Kensler et al., 2007). In our experiments, we observed that Nrf2 was increased by quercetin, a compound that could be used to prevent or decrease the oxidative stress.

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Figure 1.

A) CYP2E1 levels increased when E47 (red) hepatocytes were exposed to ethanol. CYP2E1 levels were decreased when either quercetin or catechin was added. EGCG did not decrease significantly the ethanol induced CYP2E1 levels. B) Lipid peroxidation [MDA+4HNE] was prevented in the E47 ethanol treated cells when quercetin, catechin or betaine were added. EGCG increased lipid peroxidation induced by ethanol. Mean $\pm$ SEM, n=3 (EGCG+EtOH vs. EtOH+Betaine p<0.001; EGCG+EtOH vs. EtOH+Quer p<0.001; EGCG+EtOH vs. Catech+EtOH p<0.001; EGCG+EtOH vs. control p<0.001; EGCG+EtOH vs. Ethanol p=0.002; Ethanol vs. EtOH+Betaine p=0.003; Ethanol vs. EtOH+Quer p=0.004; Ethanol vs. Catech+EtOH p=0.004; control vs. EtOH+Betaine p=0.016; control vs. EtOH+Quer p=0.017; control vs. Catech+EtOH p=0.014). C) Western blots analysis of carbonyl protein formation after ethanol and antioxidant agent treatment. 10 μ g of cytosolic proteins from each sample was used. An increase of carbonyl proteins occurred in the ethanol E47-treated cells and this smear of carbonyl proteins was decreased when quercetin was combined with ethanol treatment. However Catechin did not prevent the ethanol induced carbonyl formation. Mean $\pm$ SEM, n=3

Figure 2.

A) Human oxidative stress array results: EGCG reduced the expression of Nitric oxide synthase 2 inducible expression caused by ethanol (n=1). B) EGCG reduced the increased expression of serum/glucocorticoid regulated kinase 2 by ethanol in E47 cells. (n =1)

Figure 3.

A) EGCG, quercetin and catechin all reduced the expression of GPX4 when added to the E47 cells treated with ethanol. B) The up regulation of the expression of SOD2 by Ethanol tended to be prevented by all the antioxidants except catechin in the E47 cells. (EtOH vs. BetEtOH $p=0.028$) C) The expression of Gadd45b was not changed by ethanol alone but tended to be down regulated by all four antioxidants in the E47 cells. (EtOH E47 vs. BetEtOH E47 $p=0.047$) D) SESLN1 expression was up regulated by catechin plus ethanol compared with ethanol alone in E47 cells. E) The expression of HSP70 was in changed by compared to the control levels by ethanol and the antioxidant agents except by betaine which tended to down regulation it in the E47 ethanol treated cells. Means $\pm$ SEM, $n=4$.

Fig 4.

Study of the expression of Nrf2 protein. EtOH decreased the expression of Nrf2. This decrease was prevented by quercetin co-treatment. (Means $\pm$ SEM, $n=3$)

Table 1

Summary of cells experiments.

For Both Types of cells	Plates 100 mm	Plates 100 mm
C34 and E47	RNA	Western
Control	4	4
EtOH 100 mM	4	4
Quercetin 25 ug/ml	4	4
Quercetin + Etoh	4	4
EGCG 100 uM	4	4
EGCG + EtOH	4	4
Catechin 50 uM	4	4
Catechin + EtOH	4	4
Betaine 2 mM	4	4
Betaine + EtOH	4	4