EGCG attenuates pro-inflammatory cytokines and chemokines production in LPS-stimulated L02 hepatocyte

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Endotoxin lipopolysaccharide (LPS) plays an important role in the acceleration of inflammatory reaction of hepatitis as the second attack. Compounds that can prevent inflammation by targeting LPS have potential therapeutic clinical application. Epigallocatechin-3-gallate (EGCG) has potent hepatocyte-protective effect and mild anti-hepatitis virus function. Here, we investigated whether EGCG attenuated the severity of inflammatory response in LPS-stimulated L02 hepatocytes. L02 hepatocytes were pretreated with EGCG for 2 h, then stimulated by LPS at 250 ng/ml. The expression levels of chemokine regulated upon activation normal T-cell expressed and secreted (Rantes) and monocyte chemotactic protein-1 (MCP-1), pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interferon-γ, adhesion molecule intercellular adhesion molecule-1 (ICAM-1), oxidant stress molecules nitric oxide (NO), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-2 (MMP-2) were tested by enzyme-linked immunosorbent assay. The expression of total extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2 (p-ERK1/2), p-AKT, total p38, phospho-p38 (p-p38), total p65 and phospho-p65 (p-p65), IκBα, phospho-IκBα (p-IκBα) and TNF receptor associated factor 2 were tested by western blot analysis. Our results showed that pre-treatment with EGCG could significantly reduce the production of TNF-α, Rantes, MCP-1, ICAM-1, NO, VEGF, and MMP-2 in LPS-stimulated L02 hepatocytes in a dose-dependent manner. The effect of EGCG may be related to the inhibition of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways by down-regulation of p-IκBα, p65, p-p65, p-p38, p-ERK1/2, and p-AKT. These results indicate that EGCG suppresses LPS-induced inflammatory response and oxidant stress and exerts its hepatocyte-protective activity partially by inhibiting NF-κB and MAPK pathways.

Keywords LPS; EGCG; L02 hepatocytes; NF-κB pathway; MAPK pathway

Introduction

Chronic hepatitis is a serious health problem worldwide and a major risk factor for the development of liver cirrhosis and hepatocellular carcinoma. Liver plays an important role in clearing enterogenous bacteria and endotoxin lipopolysaccharide (LPS) [1]. The intestinal bacteria and their metabolites shift parenteral organs and over-activate immune system when liver function is severely damaged, thus leading to hepatocyte necrosis and accelerated disease progression.

Owing to increased production and absorption of gut-derived LPS, liver mononuclear macrophage system function decreases and serum LPS level significantly increases, leading to endotoxemia in chronic severe hepatitis patients. The level of endotoxin is significantly higher in cirrhotic patients and the endotoxin level is positively correlated with Child Pugh classification. Interestingly, the endotoxin levels and the incidence of complications [including upper gastrointestinal bleeding, ascites, hepatic encephalopathy, and spontaneous bacterial peritonitis (SBP)] are significantly lower after Bifidobacterium three capsules treatment for 8 weeks [2]. Therefore, the compounds that can prevent inflammation induced by LPS has potential therapeutic application in the reduction of hepatitis complications and attenuation of disease progression in patients with hepatitis.

Green tea has attracted much attention for its health benefits, epigallocatechin-3-gallate (EGCG) is the most active component of green tea and mediates most biological effects of green tea, including anti-tumor effect [3], anti-inflammatory [4], cardiovascular protection effect [5], hepatic fibrosis suppressive effect, and antioxidant activity [6].

In the present study, we tested the hypothesis of whether human hepatocytes can produce a serial of cytokines after stimulation with LPS, and whether EGCG can exert its anti-inflammatory effect in LPS-stimulated L02 hepatocytes. The
results indicate that EGCG may produce a huge decrease of cytokines secretion in LPS-stimulated L02 cells, thus pointing to EGCG as an important anti-inflammatory and hepatocyte-protective substance in hepatitis.

Materials and Methods

Drugs
EGCG was a gift from the Department of Tea in Zhejiang University. LPS (Escherichia coli 0111 : B4) was purchased from Sigma (St Louis, USA).

Cells and cell culture
Human hepatocyte cell line L02 was obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were seeded on six-well plates at a density of $1.0 \times 10^5$ per well and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, USA) supplemented with 10% (V/V) fetal bovine serum (Gibco) and antibiotics (50 IU/ml penicillin, 50 mg/ml streptomycin) (Gibco). The cells were cultured at 37°C in a humidified incubator at an atmosphere containing 5% CO₂.

Effects of LPS on L02 hepatocytes proliferation
The method to evaluate the effects of LPS on cell proliferation is based on real-time cell-based electronic sensor technology. Each well in E-plates L8 was filled with 200 μl medium, then put into the iCELLigence Station under the Apple iPad Settings application in the Wi-Fi section. Prior to putting the plates back and starting it, we put the plate for 30 min under the tissue culture hood to allow the cells to sediment homogeneously. The L02 hepatocytes were cultured in E-plates L8 for 24 h before being treated with LPS (0, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 ng/ml) for another 48 h. The impedance signal expressed as the cell index at each time point per concentration was recorded.

Cell viability assay
The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96® AQueous One Solution Cell Proliferation assay, Promega, Madison, USA) as reported previously [7]. Briefly, cells were cultured in 96-well plates and treated with EGCG (0, 1.56, 3.125, 6.25, 12.5, 25, and 50 μM) for 2 h before being incubated in LPS (250 ng/ml) for another 48 h. Then, the treated cells were incubated with 20 μl of MTS for 3 h. The absorbance was measured at 490 nm with an automatic microplate reader (Tecan, Männedorf, Switzerland). Cell viability was calculated by the following formula: cell viability (fold) = the absorbance of treated group/the average absorbance of untreated group.

Western blot analysis
The cells were exposed to EGCG at the concentrations of 0, 6.25, 12.5, 25, and 50 μM for 2 h, then incubated in the absence or presence of LPS (250 ng/ml) for 48 h before harvest. The cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (1 M Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid) with 1 mM phenylmethylsulfonyl fluoride, Halt™ Protease and Phosphatase inhibitor cocktail (Thermo, Rockford, USA) for 15 min on ice. Lysates were cleared by centrifugation at 14,000 g for 20 min at 4°C. Protein concentration was determined by the Pierce BCA protein assay kit (Thermo). The protein samples were mixed with the 5× loading buffer (4 : 1) (Bio-Rad, Hercules, USA) and heated in boiling water for 10 min. Equivalent amounts of proteins (30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA). The membranes were blocked in Tris-buffered saline with Tween 20 (TBST) containing 5% non-fat milk and incubated with antibodies at 4°C overnight, respectively. The primary antibodies included polyclonal anti-IκBα, phospho-Akt (p-Akt), total extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2 (p-ERK1/2), total p38, phospho-p38 (p-p38), total p65, and phospho-p65 (p-p65) (Cell signaling, Danvers, USA), and polyclonal anti-β-actin (Santa Cruz, Santa Cruz, USA) antibodies. After being washed three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, USA) secondary antibodies for 1 h at room temperature. After three-times wash, the blots were visualized and detected with enhanced chemiluminescence reagents (Thermo) following exposure to X-ray films.

Enzyme-linked immunosorbant assay
L02 hepatocytes were plated into six-well plates and incubated in 37°C in a humidified incubator at an atmosphere containing 5% CO₂. After being washed with PBS, L02 hepatocytes were pre-treated with increasing concentrations of EGCG (0, 6.25, 12.5, 25, and 50 μM) in DMEM for 2 h, followed by LPS (250 ng/ml) stimulation for 48 h. The cell supernatants were then collected, and the expression levels Rantes, monocye chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-2 (MMP-2) were analyzed by using commercially available enzyme-linked immunosorbant assay kits (R&D Systems Inc., Minneapolis, USA) according to the manufacturer’s instructions and the absorbance was read at 450 nm in Microplate reader (Bio-Rad). Assays were performed in duplicate. Background activity in the negative control wells was subtracted from the experimental wells.
Nitric oxide assay
L02 hepatocytes were treated as above, and the nitrite concentration in the culture medium was measured as an indicator of nitric oxide (NO) production by Griess reaction (BioVision, Milpitas, USA). Absorbance was read at 540 nm in a Microplate reader.

Statistical analysis
All of the experiments were repeated at least three times. The results are given as the mean ± standard error of the mean. Data were analyzed by using analysis of variance. Correlation between NO and VEGF was tested by the Pearson coefficient of correlation. A difference was considered significant when \( P < 0.05 \).

Results

Effects of LPS on L02 hepatocytes and the determination of LPS stimulation concentration
As shown in Fig. 1A, LPS (0–1000 ng/ml) did not effect L02 hepatocytes proliferation. With the concentration of LPS (≤250 ng/ml) increasing, the levels of nitrite and TNF-\( \alpha \) were in a straight-line increase. The production of nitrite and TNF-\( \alpha \) showed statistically significant differences among the 62.5, 125, and 250 ng/ml LPS groups (Fig. 1B,C). Therefore, 250 ng/ml (LPS) was chosen as the best concentration for stimulation.

Effect of EGCG on cell viability
In order to assess the impact of pre-treatment with EGCG on LPS-stimulated L02 cell viability and to confirm that the findings were not due to the cytotoxicity effects of EGCG, the cells were exposed to different concentrations of EGCG (0–50 \( \mu \)M) for 2 h and then incubated in LPS (250 ng/ml) for 48 h. MTS assay indicated that EGCG did not influence the cell viability of L02 hepatocytes (Fig. 2).

EGCG inhibits the production of pro-inflammatory cytokines
To analyze EGCG on chemokines expression in LPS-stimulated L02 hepatocytes, we determined the levels of Rantes and
The results showed that LPS stimulation significantly increased the expression of Rantes and MCP-1. Compared with the LPS stimulation group, EGCG pre-treatment at different concentrations significantly decreased the production of Rantes and MCP-1 ($P < 0.05$). Our results suggested that EGCG dose-dependently inhibited Rantes and MCP-1 production in LPS-stimulated L02 hepatocytes (Fig. 3A,B).

To investigate the inflammation cytokines changes in LPS-stimulated L02 hepatocytes under EGCG pre-treatment we examined the expression of ICAM-1 and TNF-$\alpha$. The results showed that LPS significantly induced the expression of ICAM-1 and TNF-$\alpha$, and EGCG inhibited ICAM-1 and TNF-$\alpha$ production in LPS-stimulated L02 hepatocytes in a dose-dependent manner (Fig. 3C,D).

We investigated the effects of EGCG on MMP-2 expression in LPS-stimulated L02 hepatocytes. Compared with the control group, the LPS group significantly increased the level of MMP-2 ($P < 0.05$). Compared with the LPS group, EGCG significantly inhibited MMP-2 production in a dose-dependent manner (Fig. 3E).

The production of NO and the expression of nitric oxide synthase (NOS) in treated L02 hepatocytes were evaluated. Our results showed that LPS stimulation significantly increased the level of NO. Compared with the LPS group, EGCG treatment significantly decreased the production of NO ($P < 0.05$) in a dose-dependent manner (Fig. 4A). Meanwhile, the expression of inducible NOS (iNOS) was
assayed after the cells were pre-treated with the indicated concentrations of EGCG for 2 h followed by LPS (250 ng/ml) or PBS for 48 h. As demonstrated in Fig. 4B, the experimental groups showed a mild increase in iNOS production when compared with the control. However, no significant difference was observed.

The levels of VEGF were also detected in treated L02 hepatocytes. As shown in Fig. 4C, compared with the control group, the LPS group significantly increased the level of VEGF ($P < 0.05$). Compared with the LPS group, EGCG pre-treatment inhibited VEGF production in a dose-dependent manner ($P < 0.05$).

EGCG had a minor increase effect on iNOS production, and NO was catalyzed by iNOS, but EGCG pre-treatment significantly inhibited the expression of NO. Hence, we speculated that other molecules might also be involved in the production of NO. By binding to its receptors, VEGF initiates the signaling cascades that lead to NO production [8]. Therefore, we evaluated the correlation of VEGF with NO, and found that the amount of NO was positively correlated with that of VEGF ($r = 0.914, P < 0.01$) (Fig. 4D), suggesting that the inhibitory effect of EGCG on NO production might associate with VEGF other than iNOS.

**EGCG exerts its hepatocyte-protective activity by inhibiting nuclear factor-$\kappa$B signaling pathway**

A variety of stress stimuli have been demonstrated to cause the activation of nuclear factor-$\kappa$B (NF-$\kappa$B), such as LPS, TNF-$\alpha$, interleukin-1, ceramide, phorbol myristate acetate, and hydrogen peroxide [7]. Therefore, we detected the expression of TNF receptor associated factor 2 (TRAF2), IkB$\alpha$, p65, and p-p65 to confirm whether EGCG regulated inflammation via the NF-$\kappa$B signaling pathway (Fig. 5). Compared with the control group, the LPS group significantly increased the level of TRAF2. However, pre-treatment with EGCG did not affect LPS-induced TRAF2 expression. Compared with the control group, the LPS group significantly increased the level of p-IkB$\alpha$ and p-p65. Compared with the LPS group, EGCG pre-treatment significantly decreased the production of p-IkB$\alpha$ and p-p65. Our results suggest that EGCG administration inhibits p-IkB$\alpha$ and p-p65 expression in a dose-dependent manner in LPS-stimulated L02 hepatocytes.

**EGCG exerts its hepatocyte-protective activity by inhibiting MAPK signaling pathway**

Previous studies have shown that p38$\alpha$-deficient hepatocytes are not sensitive to LPS/TNF toxicity [9]. To confirm whether...
MAPK activation was inhibited in EGCG pre-treated L02 hepatocytes, the phosphorylation of ERK1/2 and p38 were examined by western blot analysis. As demonstrated in Fig. 6, EGCG significantly inhibited the expression of p-p38, and dose-dependently reduced the level of p-ERK1/2 in LPS-stimulated L02 hepatocytes, but it did not influence the total protein p38, ERK1, and ERK2 expression. Compared with the control group, the LPS group significantly increased the level of p-p38 and p-ERK1/2. Compared with the LPS group, treatment with EGCG before stimulation with LPS groups significantly decreased the expression of p-p38 and p-ERK1/2.

A previous study has reported that insulin reduces LPS-induced inflammation in mice in PI3K/Akt-dependent manner without affecting blood glucose levels [10]. To confirm the role of PI3K/Akt in LPS-stimulated L02 hepatocytes, the expression of p-AKT was detected by western blot analysis. Pre-treatment with EGCG significantly reduced the expression of p-AKT dose-dependently in LPS-stimulated L02 hepatocytes (Fig. 7). Compared with the control group, the LPS group significantly increased the level of p-AKT. Compared with the LPS group, treatment with EGCG before stimulation with LPS groups significantly decreased the expression of p-AKT.

Discussion

Liver is the major organ to get rid of LPS. High level of LPS will lead to endotoxemia, which induces viral hepatitis related complications and accelerates hepatitis progression. The expression of pro-inflammation cytokines will be increased after stimulation with LPS, and inflammatory cytokines may activate signaling molecules, eventually leading to liver damage. We look forward to simulate the body’s systemic process to study the ultimate effects of LPS on the body, instead of LPS’s direct effect on the signaling pathways. Therefore, we detected the cytokines transcription after LPS stimulation for
Our results suggested that LPS induced the expression of TNF-α, Rantes, MCP-1, ICAM-1, NO, VEGF, and MMP-2 in LPS-stimulated L02 hepatocytes, which explained why endotoxemia exacerbates the progression and severity of viral hepatitis at molecular level. Pre-treatment with EGCG could significantly reduce the production of TNF-α, Rantes, MCP-1, ICAM-1, NO, VEGF, and MMP-2 in LPS-stimulated L02 hepatocytes in dose-dependent manner, indicating that EGCG is hepatocyte-protective and may be used to regulate over-activated inflammatory reaction of hepatitis.

A previous study has concluded that the anti-inflammatory mechanism of green tea polyphenols contributes to down-regulation of TNF-α gene expression by blocking NF-κB activation [11]. Exposure to LPS can induce hepatitis and acute lung injury in mice and lead to a large increase of TNF-α production by macrophage/Kupffer cells [12]. Our results are in agreement with the previous study showing that EGCG can inhibit LPS-induced TNF-α production in mice [11].

EGCG is found to inhibit iNOS protein in activated macrophages [11], which produces large amounts of NO by endotoxin and/or cytokines stimulation and plays an important role in toxin-induced liver injury [13]. However, our results suggested that pre-treatment with increased EGCG induced an increase of iNOS secretion in LPS-stimulated L02, but no statistical significance was observed, which may be due to the different experimental cells or medicine used.

Levels of VEGF protein in tissue expression and circulating tend to increase during acute and chronic hepatitis, while

Figure 6. Effects of EGCG on the MAPK signaling pathway in LPS-induced L02 hepatocytes  The cells were exposed to EGCG at the concentrations of 0, 6.25, 12.5, 25, and 50 μM for 2 h, then incubated in the presence of LPS (250 ng/ml) or PBS for 48 h before harvest. (A) The expression of p38, p-p38, ERK, ERK2, p-ERK1, and p-ERK2 were determined by western blot analysis. (B–G) The quantification of the protein expression by densitometry. (B), p38/β-actin; (C), p-p38/β-actin; (D), ERK1/β-actin; (E), ERK2/β-actin; (F), p-ERK1/β-actin; (G), and p-ERK2/β-actin.
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EGCG attenuates pro-inflammatory reaction in LPS-treated L02 cells

Figure 7. Effect of EGCG on LPS-induced phosphorylation of AKT in L02 hepatocytes (A) The cells were pre-treated with EGCG at the concentrations of 0, 6.25, 12.5, 25, and 50 μM for 2 h, then incubated in LPS (250 ng/ml) or PBS for 48 h. (B) The quantification of the protein expression by densitometry.

it decreases during cirrhosis [14]. In addition, higher level of VEGF in ascites of SBP patients in contrast to non-infected cirrhotic patients was observed [15]. Ascites of SBP patients contains larger amounts of cytokines than that of cirrhotic patients without peritonitis [16]. Therefore, VEGF may influence the increased permeability of hepatic microvessels and induce over-activation of inflammatory response.

The relationship between the Rantes gene polymorphisms and the clinical course of HBV infection has been analyzed and used to evaluate the genetic background of the affected individual as well as disease progression in a Saudi population [17]. Concentration of chemokine Rantes is higher in HBV infected patients than in the controls [18]. Besides, significant increases of the levels of Rantes, TNF-α, and NO in Hepatitis C virus-infected patients are observed [19]. Another study has shown that MCP-1 plasma levels are higher in both alcoholic liver disease and alcoholic hepatitis patients than in the controls, and alcoholic hepatitis patients have significantly higher hepatic expression of MCP-1 [20]. Our results indicated that EGCG inhibited the expression of Rantes and MCP-1, which in turn inhibited the migration of inflammatory cells into the liver, thus reducing liver injury severity. It is interesting to speculate that EGCG may exert its hepatocyte-protective activity in hepatitis B and/or hepatitis C.

MMP-2 expression was negative or very low in the livers of normal, cirrhosis, and chronic moderate hepatitis; whereas there is a strong expression in the livers of acute liver injury of alcoholic hepatitis and acute viral hepatitis, indicating that MMP-2 expression, to a certain extent, reflects inflammatory response in the liver. Our results suggested that EGCG inhibited liver fibrosis and inflammation through reduced MMP-2 secretion in injured hepatocytes to some degree, since hepatic fibrosis is a prosthetic response to chronic liver injury, still a stage of development of chronic hepatopathy to liver cirrhosis. In addition, we examined the effect of EGCG on the production of interferon-γ in LPS-stimulated L02 cells, but no significant difference was observed.

Previous studies have demonstrated that NF-κB regulates the expression of MMP-2 [21], TNF-α [11], iNOS [22], and ICAM-1 [23], which indicated that NF-κB might mediate LPS-induced inflammatory response.

NF-κB, is a widely distributed transcription factor, which is involved in many physiological processes, including inflammation, cellular proliferation, and cancer [7]. NF-κB exists in the cytosol as a heterotrimer that usually consisted of its p50 and p65 subunits, binds to its inhibitory protein IκB, in particular IκBα. Upon activation, IκBα is phosphorylated, and degraded by IκB kinases, followed by translocating into the nucleus to activate the expression of a set of NF-κB responsive genes.

Pre-treatment with EGCG dose-dependently decreased p-p65 and p-IκBα level in LPS-stimulated L02 hepatocytes, suggesting that the anti-inflammatory property of EGCG was at least in part mediated by inhibiting the NF-κB pathway. Previous studies have shown that LPS-mediated IκB degradation was blocked by the EGCG [22,24], which indicated that the anti-inflammatory effect of EGCG is different among different cells.

To better understand the molecular mechanisms underlying the anti-inflammatory effect of EGCG, we further explored the effect of EGCG on MAPK signaling pathways in LPS-stimulated L02 hepatocytes. ERK1/2 and p38 are the major members of MAPKs and are associated with cellular oxidative stress, inflammation, proliferation, and migration.

A previous study has found that EGCG could inhibit LPS-induced phosphorylation of ERK1/2, JNK, and p38 in bone marrow-derived macrophages [24]. Intestinal endotoxemia accompanied by cirrhosis may be an important mechanism in the development of hepatopulmonary syndrome in rats. The major mechanism mediating the pathological alterations of hepatopulmonary syndrome is the overproduction of TNF-α due to endotoxin stimulation of Kupffer cells via MAPK pathway [25]. Our results showed that EGCG dose-dependently inhibited LPS-induced phosphorylation of p38 and ERK1/2 in LPS-stimulated L02 hepatocytes, which suggested that the inhibitory effect of EGCG on the expression of inflammatory response may involve the suppression of MAPK signaling pathways.

In conclusion, the protective effect of EGCG in LPS-stimulated L02 hepatocytes is associated with the inhibition
of several inflammatory mediators, including TNF-α, Rantes, MCP-1, ICAM-1, NO, VEGF, and MMP-2, and may involve the NF-κB and MAPK signaling pathways. In the future, we will further study whether EGCG inhibits pro-inflammatory cytokines and chemokines production in LPS-treated mouse. Taken together, EGCG might be a promising agent for the treatment of hepatic over-active inflammatory response.

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