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Epigallocatechin-3-gallate attenuates microcystin-LR-induced apoptosis in human umbilical vein endothelial cells through activation of the NRF2/HO-1 pathway☆

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ABSTRACT

Our previous study showed that the tea extract, epigallocatechin-3-gallate (EGCG), protects against microcystin-LR (MC-LR) -mediated apoptosis of human umbilical vein endothelial cells (HUVECs); however, the mechanism underlying MC-LR-induced HUVEC apoptosis remains incompletely understood. In this study, we investigated whether the nuclear factor erythroid-like 2 (NRF2)/heme oxygenase-1 (HO-1) pathway, which regulates antioxidant transcriptional regulation of oxidative stress and apoptosis, is involved in this process. Mitochondrial membrane potential (MMP) and caspase-3/-9 activities were evaluated in HUVECs by JC-1 staining and colorimetric activity assay, and a DCFH-DA fluorescent probe assay was used to quantitate reactive oxygen species (ROS) generation. The effects of MC-LR, EGCG, NF2, and HO-1 on HUVEC apoptosis were explored by western blotting and small interfering RNA (siRNA) analyses. MC-LR treatment downregulated HUVEC mitochondrial membrane potential, and decreased levels of cytochrome *c* release and activated caspase-3/-9, ROS generation, consequently inducing HUVEC apoptosis. EGCG treatment attenuated MC-LR-mediated HUVEC oxidative stress and mitochondria-related apoptosis. EGCG induced NRF2/HO-1 expression and activation in MC-LR treated HUVECs, while downregulation of NRF2/HO-1 by specific siRNAs revealed that NRF2/HO-1 signaling was involved in EGCG attenuation of MC-LR-induced HUVEC apoptosis. Our findings indicate that EGCG treatment protects against MC-LR-mediated HUVEC apoptosis via activation of NRF2/HO-1 signaling.

1. Introduction

Endothelial dysfunction is predictive of atherosclerosis (Kershaw et al., 2017), cardiovascular events (Park and Park, 2015), and the long-term clinical outcomes of such conditions (Guazzi et al., 2015; Corte et al., 2016). Endothelial cell apoptosis is key in induction of vascular endothelial injury and the development of cardiovascular disease (CVD) (Tang et al., 2017; Huang et al., 2017). Approaches designed to improve endothelial function may have therapeutic value for prevention and treatment of atherosclerotic disease.

Microcystin-LR (MC-LR) is belong to a group of cyclic heptapeptide cyanotoxins in eutrophic freshwaters. MC-LR is a risk factor for cellular dysfunction, which can induce cognitive impairment (Cai et al., 2015) and cell death (Shuai et al., 2017; D'Mello et al., 2017; Huang et al., 2016). Our previous studies indicated that MC-LR stimulates intracellular reactive oxygen species (ROS) generation in vascular cells, which induces endothelial apoptosis and cellular dysfunction (Shi et al., 2015); however, the mechanisms underlying MC-LR-induced endothelial cell apoptosis signaling events remain to be fully elucidated.

Nuclear factor erythroid-like 2 (NRF2) is involved in defense of cells against oxidative stress (Zhang et al., 2017; Wu et al., 2017; Wang et al., 2015), which induces NRF2 activation and translocation to the nucleus, where its accumulation leads to upregulation of

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antioxidant enzyme transcription, such as superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) (Zenkov et al., 2017). Here we investigated the effect of NRF2/HO-1 on MC-LR-mediated endothelial injury and explored its molecular mechanisms.

Epigallocatechin-3-gallate (EGCG), a compound isolated from tea, has anti-inflammatory, anti-oxidative, and anti-angiogenic effects, both *in vitro* and in vivo (Bae et al., 2017; Borse et al., 2017; Chen et al., 2017; Fu et al., 2017). EGCG can enhance cancer cell apoptosis (Modernelli et al., 2015; Wang et al., 2017), and there is evidence that it ameliorates the progression of atherosclerotic lesions and vascular injury (Chyu et al., 2004). Although our previous study provided important experimental evidence supporting the conclusion that EGCG has a protective effect on endothelial cells (Shi et al., 2017), it remains unclear whether EGCG ameliorates MC-LR-induced damage to human endothelial cells via effects on mitochondrial dysfunction and/or NRF2 signaling.

In this study, we investigated the effects of EGCG on MC-LR-induced injury to human umbilical vein endothelial cells (HUVECs), and their underlying mechanisms. Our results suggest that EGCG can attenuate MC-LR-mediated HUVEC injury through activation of NRF2 signaling.

2. Materials and methods

2.1. Chemicals and reagents

Microcystin-LR (purity > 95%) was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. EGCG was obtained from Sigma-Aldrich (St. Louis, MO, USA). All antibodies, including primary and horseradish peroxidase-conjugated secondary antibodies, were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

HUVECs were obtained from Allcells Technology (Shanghai, China) and seeded in complete medium (HUVEC-004, AllCells Technology) in a humidified atmosphere of 5% CO_2 at 37 °C. HUVECs were treated with EGCG (0–50 μM) and/or MC-LR (40 μM) for 24 h.

2.3. Detection of apoptotic cells by TUNEL staining

After washing with phosphate-buffered saline, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich). Then, apoptotic cells were detected using a TUNEL fluorescence kit (Roche, Indianapolis, IN, USA) by incubation at 37 °C for 1 h, following the manufacturer's protocols. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Stained cells were examined under a fluorescence microscope (LSM710; Carl Zeiss Microscopy GmbH, Jena, Germany).

2.4. Hoechst 33342 staining

Hoechst 33342 staining was used to detect HUVEC apoptosis, according to the manufacturer's instructions. Briefly, HUVECs were fixed with 4% paraformaldehyde for 30 min at room temperature, then stained with Hoechst 33342 for 20 min. After washing three times with serum-free DMEM, observation of cell morphology was performed using a fluorescence microscope (LSM710; Carl Zeiss Microscopy GmbH, Jena, Germany).

2.5. Detection of cytoplasmic and mitochondrial ROS

The DCFH-DA staining assay was used to measure cytoplasmic ROS generation. HUVEC cells were incubated with DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA, 10 μmol/L) at 37 °C for 30 min. For detection of mitochondrial ROS production, HUVECs were incubated with MitoSOX Red mitochondrial superoxide indicator (Invitrogen Life Technologies, Carlsbad, CA, USA, 5 μmol/L) at 37 °C for 10 min. Images were collected using a fluorescence microscope (CKX4, Olympus, Tokyo, Japan) and analyzed with Image-Pro software.

2.6. Measurement of mitochondrial membrane potential (MMP, $\Delta\Psi m$)

Mitochondrial membrane potential ($\Delta\Psi m$) was determined using JC-1 dye (Thermo Fisher Scientific Inc., MA, USA). Mitochondria were extracted from HUVECs using a mitochondria isolation kit (Sigma-Aldrich, St. Louis, MO, USA), then incubated with JC-1 (10 μg/mL) for 15 min at 37 °C in the dark. Subsequently, cells were visualized using a fluorescence microscope (CKX4, Olympus, Tokyo, Japan) (Kumar et al., 2014).

2.7. Caspase activity assay

Cells were harvested and lysed, and caspase-3 and -9 activities measured using a colorimetric activity assay kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions.

2.8. Measurement of cytochrome C (cytc) release

The Quantikine M Cytochrome C Immunoassay kit (R&D Systems, Minneapolis, MN, USA) was used to determine Cytc levels in cytosolic and mitochondrial fractions, as previously described (Toniolo et al., 2015).

2.9. RNAi-mediated silencing of NRF2

Cells were transfected with siRNAs targeting NRF2 or heme oxygenase-1 (HO-1) (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, USA), following the manufacturer's instructions. Non-specific non-targeting siRNA (scrambled) was used as a negative control. Cells were examined by western blotting 48 h after transfection.

2.10. Western blotting

Western blotting was performed as previously described. Proteins lysates were extracted using RIPA buffer containing protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., MA, USA). Subsequently, 30 μg protein aliquots were separated by 10% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk for 1 h at room temperature, then incubated with primary antibodies overnight at 4 °C. Subsequently, membranes were exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h at 37 °C and incubated with an ECL reagent system (Thermo Scientific). Specific bands were quantified using Image J Software (NIH, Bethesda, MD, USA).

2.11. Quantitation of NRF2 activity by ELISA

NRF2 activity levels were determined using a TransAM NRF2 assay Kit (Active Motif, Carlsbad, CA), according to the