Life Sciences 86 (2010) 410–415

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Epigallocatechin-3-gallate inhibits interleukin-6- and angiotensin II-induced production of C-reactive protein in vascular smooth muscle cells

Ning Peng [a,b], Jun-tian Liu [a,*], Fang Guo [a], Rui Li [b]

[a] Department of Pharmacology, Xi'an Jiaotong University School of Medicine, Xi'an, China
[b] People's Hospital of Shaanxi Province, Xi'an, China

ARTICLE INFO

Article history:
Received 14 August 2009
Accepted 14 January 2010

Keywords:
Epigallocatechin-3-gallate
Interleukin-6
C-reactive protein
Angiotensin II
Atherosclerosis
Reactive oxygen species

ABSTRACT

Aims: Extensive research suggests that atherosclerosis is an inflammatory disease and that epigallocatechin-3-gallate (EGCG) is able to inhibit the formation and development of atherosclerosis. However, the mechanisms of action of EGCG against atherosclerosis are still unclear. Therefore, the effect of EGCG on interleukin-6 (IL-6)- and angiotensin II (Ang II)-induced CRP production in vascular smooth muscle cells (VSMCs) was studied to provide experimental evidence for its anti-inflammatory and anti-atherosclerotic actions.

Main methods: Rat VSMCs were cultured, and IL-6 (10^{-7} M) and Ang II (10^{-7} M) were used as stimulants for CRP generation. The CRP concentration in the supernatant was measured with ELISA, and mRNA and protein expression of CRP was assayed with RT-qPCR and immunocytochemistry, respectively. The production of reactive oxygen species (ROS) and superoxide anion (O_2^-) was detected with ROS and O_2^- assay kits, respectively.

Key findings: The results showed that both IL-6 and Ang II increased CRP levels in the supernatant of VSMCs and induced mRNA and protein expression of CRP in VSMCs, whereas pretreatment of the cells with EGCG (1×10^{-6} M, 3×10^{-6} M, 10×10^{-6} M) significantly inhibited IL-6- and Ang II-induced production and expression of CRP in VSMCs in a concentration-dependent manner. Additionally, Ang II stimulated O_2^- and ROS generations in VSMCs, and EGCG decreased the Ang II-induced increase of O_2^- and ROS in a concentration-dependent fashion.

Significance: These results suggest that EGCG plays an anti-inflammatory role via inhibiting IL-6- and Ang II-induced CRP secretion, as well as the Ang II-induced generation of O_2^- and ROS in VSMCs, which contributes to its anti-atherosclerotic action.

Introduction

The inflammatory response plays an important role in the onset, development and evolution of atherosclerotic lesions (Libby 2002). As an exquisitely sensitive marker of inflammation, C-reactive protein (CRP) has emerged as an important predictor of cardiovascular events (Jialal et al. 2004; Danesh et al. 2004). Large studies support the proatherogenic effects of CRP via injuring endothelial cells, promoting monocyte–macrophage accumulation, and stimulating the migration and proliferation of vascular smooth muscle cells (VSMCs). Further investigations have shown that CRP causes the expression of monocyte chemoattractant protein-1, interleukin-6 (IL-6) and inducible nitric oxide synthase through activating the MAPK-c-Fos/c-Jun-AP-1 pathway in VSMCs. Recently, a study also showed that human coronary artery smooth muscle cells are able to generate CRP after being stimulated with inflammatory cytokines (IL-1β, IL-6 and tumor necrosis factor-α), which raises the possibility that human coronary artery smooth muscle cells may be a source of locally produced CRP in the arterial wall (Calabró et al. 2003). Our previous study also found that angiotensin II (Ang II) is capable of inducing CRP generation in VSMCs, which strengthens the role of Ang II in the inflammatory process in atherosclerosis (Peng et al. 2007). Additionally, Ang II can induce the generation of reactive oxygen species (ROS) and superoxide anion (O_2^-). As signaling molecules, ROS play crucial roles not only in Ang II-mediated pathophysiological processes, but also in atherosclerosis via mediating specific cellular responses, such as CRP and IL-6 generation (Griendling et al. 2000).

Epigallocatechin-3-gallate (EGCG), mainly contained in green tea and red wine, has received attention as a prospective dietary

* Corresponding author. Postbox 58, Xi'an Jiaotong University School of Medicine, 76 West Yanta Road, Xi'an 710061, P. R. China. Tel./fax: +86 29 82655188.
E-mail address: ljt@mail.xjtu.edu.cn (J. Liu).

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doi:10.1016/j.lfs.2010.01.010

intervention in cardiovascular diseases (Zaveri 2006; L'Allemain 1999; Miura et al. 2000). Experimental studies have confirmed that green tea catechins reduce atherosclerotic lesions in animal models of hyperlipidemia (Chyu et al. 2004), and that EGCG suppresses the migration of human VSMCs and the low-density lipoprotein-induced proliferation of rat VSMCs. In addition, EGCG prevents pathogenesis of cardiovascular diseases by acting as a scavenger of ROS (Wei et al. 2004; Cheng et al. 2005). Our past work also demonstrated that EGCG possesses an anti-inflammatory property in microglial cells (Li et al. 2006). Since chronic inflammation in the artery wall plays a central role in atherogenesis, we hypothesized that EGCG possibly produces an anti-inflammatory effect on vascular cells. Hence, the present study observed the inhibitory effects of EGCG on the IL-6- and Ang II-induced generation of CRP in VSMCs.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and Trizol reagents were purchased from GIBCO BRL (Carlsbad, CA, USA). IL-6 was from R&D systems (USA). Ang II and EGCG were from Sigma (USA). The rat CRP ELISA kit was produced by Alpha Diagnostics International (San Antonio, TX, USA). CRP antibody, α-actin antibody and the ABC kit for immunocytochemical staining were obtained from Beijing Biosynthesis Biotechnology Co., LTD (Beijing, China). The ROS and superoxide anion assay kits were purchased from the Beyotime Institute of Biotechnology (Shanghai, China).

Cell culture

VSMCs were isolated from the thoracic aorta of Sprague–Dawley rats as described previously (Griendling et al. 1991) and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 until the cells exhibited the typical "hill and valley" growth morphology. Then, the cells were verified by immunocytochemical staining for α-actin. VMSCs at passage 3–10 were used for the experiments. The culture medium was changed to DMEM supplemented with 0.1% FBS for an additional 24 h before the experiments.

Enzyme-linked immunosorbent assay (ELISA)

Confluent VSMCs grown in 6-well plates were pretreated with different concentrations of EGCG (1×10^{-6} M, 3×10^{-6} M, 10×10^{-6} M) for 30 min and stimulated by IL-6 (10^{-7} M) or Ang II (10^{-7} M) for 24 h. Then, the conditioned medium was collected and concentrated (~20 fold) using centrifugal filter units (Millipore, Bedford, MA, USA), and the CRP concentration was assayed by an ELISA kit specific for rat CRP according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The mRNA level of CRP was quantitatively measured by RT-qPCR. In brief, total cellular RNA was extracted by the Trizol reagent and quantified by measuring the absorbance at 260 and 280 nm. The total RNA purity (A_{260}/A_{280}) was between 1.6 and 1.9. The quality of RNA was confirmed by ethidium bromide staining after 1% agarose gel fractionation. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using the Revert Aid™ First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) following the manufacturer's protocol. Real-time polymerase chain reaction with SYBR was performed with a SuperScript™III Platinum® Two-Step qRT-PCR kit (Invitrogen, USA) on an ABI Prism 7000 sequence detection PCR system (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Specific cDNA from the reverse transcriptase reaction product was amplified using a primer pair specific for rat CRP (sense primer: 5′-CACAACAGTCAGTCAAGG-3′, antisense primer: 5′-GTGCTATCTCCAGAACAG-3′), which yielded a fragment of 142 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA served as an internal control (sense primer: 5′-GCCTTCTCCATGGTGGTGAA-3′, antisense primer: 5′-GGTCGGTGTGAACGGATTTG-3′). The reaction conditions were 2 min at 50 °C, 2 min at 95 °C, 15 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C for 45 cycles. In all of the experiments, control reactions were performed by substituting sterile nuclease-free water for the RNA template in the reaction. The specificity of the RT-PCR products was confirmed by melting curves and visualization on 1% TAE agarose gel with ethidium bromide staining. The relative amount of each mRNA was normalized to the housekeeping gene (GAPDH) mRNA. Quantitative data of relative gene expression were determined by the comparative Ct method ($2^{-\Delta\Delta Ct}$), as described by the manufacturer.

Immunocytochemical staining

The stimulated VSMCs were moved to cover slips and mounted on slides. All of the slides were immersed in 3% H_2O_2 in methanol for 45 min at room temperature to block the activity of endogenous peroxidases. The sections were then incubated with 10% normal goat serum for 1.5 h and reacted with CRP antibody (1:300) overnight at 4 °C. After being washed several times with phosphate-buffered saline (pH 7.4), the sections were incubated with peroxidase-conjugated secondary antibody for 60 min at room temperature. The immunological staining was developed with an ABC kit.

Measurement of intracellular ROS and O_2^-

ROS generation in VSMCs was monitored with the oxidation-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), as previously described (Bass et al. 1983). In brief, subconfluent VSMCs were seeded on poly-L-lysine coated glass cover slips (8×8 mm) in 24-well plates and incubated for 20 min at 37 °C with 10^{-5} M H_2DCF-DA in serum-free medium. Then, the cells were pretreated with different concentrations of EGCG (1×10^{-6} M, 3×10^{-6} M, 10×10^{-6} M) for 30 min before being stimulated with Ang II (10^{-7} M). Subsequently, the cells were imaged with a laser scanning confocal microscope (Leica TCS SP2, Germany) in a dark environment every 2 min for 10 min after the specimen was mounted on the object stage. Fluorescence images were acquired at excitation and emission wavelengths of 488 and 525 nm, respectively. The fluorescence intensity of an experimental field comprising 15–20 cells of each group was measured and analyzed with Image-pro plus software. The relative fluorescence intensity was taken as the average of values from four repeated experiments.

Intracellular O_2^- was detected using a superoxide anion assay kit from the Beyotime Institute of Biotechnology (Shanghai, China), which is based on O_2^- deoxidizing WST-1[2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] to a soluble and colored substance showing an absorbance at 450 nm. Confluent VSMCs grown in 96-well plates were pretreated with different concentrations of EGCG (1×10^{-6} M, 3×10^{-6} M, 10×10^{-6} M) for 30 min prior to stimulation with Ang II (10^{-7} M). Then, the cells were washed with PBS, and 200 μL of O_2^- assay working solution was added into the cells for further incubation for 3 min at 37 °C according to the manufacturer's instructions. Next, phorbol myristate acetate was added to stimulate the cells for 30 min. Finally, the absorbance was detected at 450 nm every 2 min for 10 min.

Statistical analysis

All values are expressed as mean ± S.D. Statistical significance between groups was assessed by using one-way ANOVA, followed by