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Epigallocatechin-3-gallate inhibits VCAM-1 expression and apoptosis induction associated with LC3 expressions in TNFα-stimulated human endothelial cells

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ABSTRACT

Tumor necrosis factor alpha (TNF-α) promotes the expression of adhesion molecules and induces endothelial dysfunction, a process that can lead to atherosclerosis. Green tea consumption can inhibit endothelial dysfunction and attenuate the development of arteriosclerosis. The purpose of this study was to examine whether epigallocatechin-3-gallate (EGCG) prevents TNF-α-dependent endothelial dysfunction. Here, we compared the regulatory effects of the green tea components EGCG and L-theanine against TNF-α-induced stimulation of adhesion molecule expression and apoptosis induction, which is associated with autophagy. Monocytic cell adhesion to human endothelial cells was measured using a fluorescently-labeled cell line, U-937. Caspase 3/7 activity was examined with a fluorescent probe and fluorescence microscopy. In addition, we analyzed the expression of several genes by RT-PCR. TNF-α-modulation of LC3 and VCAM1 protein levels were investigated by Western blot (WB). TNF-α induced adhesion of U937 cells to endothelial cells, and gene expression associated with adhesion molecules and apoptosis. On the other hand, EGCG and L-theanine inhibited TNF-α-induced adhesion of U937 cells to endothelial cells and inhibited increases in *ICAM1*, *CCL2* and *VCAM1* expression. Furthermore, EGCG and L-theanine inhibited TNF-α-induced apoptosis-related gene expression (e.g., *CASP9*), and caspase activity while inhibiting TNFα-induced VCAM1, LC3A and LC3B protein expression. Meanwhile, treatment of endothelial cells with autophagy inhibitor 3-methyladenine (3-MA) blocked EGCG-induced expression of *CASP9*. Together, these results indicate that EGCG can modulate TNF-α-induced monocytic cell adhesion, apoptosis and autophagy. We thus conclude that EGCG might be beneficial for inhibiting TNF-α-mediated human endothelial disorders by affecting LC3 expression-related processes.

Abbreviations

EC	epicatechin
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin-3-gallate
eNOS	endothelial nitric oxide synthase
HO-1	heme oxygenase-1
ICAM1	intercellular adhesion molecule-1
JNK	c-Jun N-terminal kinase
HUVEC	human umbilical vein endothelial cells
3-MA	3-Methyladenine
MCP1	monocyte chemoattractant protein-1
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
ROS	reactive oxygen species
TNF-α	tumor necrosis factor alpha
VCAM1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

Introduction

In epidemiological studies, green tea (*Camellia sinensis*) consumption was shown to be related to decreases in cardiovascular disease morbidity and mortality (Kuriyama 2008). Epigallocatechin-3-gallate (EGCG) (Fig. 1A) is a major polyphenol present in green tea that has beneficial effects in preventing cardiovascular disease (Wolfram 2007). The chemical structure of epigallocatechin-3-gallate is shown in Fig. 1A. EGCG appears to affect the relationship between endothelial

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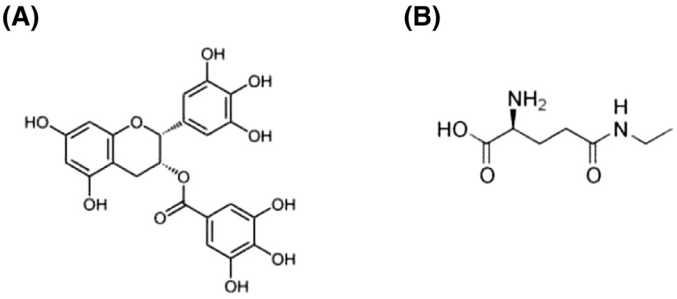

Fig. 1. Chemical structure of epigallocatechin-3-gallate and L-theanine. (A) Epigallocatechin-3-gallate (EGCG) and (B) L-theanine.

dysfunction and diseases involving insulin resistance, including diabetes and metabolic syndrome, and in turn their vascular complications (Wolfram et al. 2006; Bose et al. 2008). For instance, EGCG upregulates nitric oxide (NO) levels by activating Fyn/PI3K/Akt/endothelial nitric oxide synthase (eNOS) that affects vascular endothelial cells, which then results in vasodilation (Kim et al. 2007). In addition, EGCG enhances production of heme oxygenase-1 (HO-1) by vascular endothelial cells, which can reduce hypertension and endothelial dysfunction in spontaneously hypertensive rats (Pullikotil et al. 2012). On the other hand, green tea also includes trace amounts (0.5–2.0% of the dry weight of both green and black teas) of L-theanine, an amino acid (γ-glutamylethylamide) and glutamate derivative (Nobre et al. 2008, Fig. 1B). L-theanine relaxes blood vessels, acts as a mood stabilizer (Einother and Martens 2013) and provides neuronal protection (Kakuda et al. 2000). In addition, L-theanine reduces blood pressure that is elevated by high-stress responses associated with physical or psychological stress in humans (Yoto et al. 2012). L-theanine activates eNOS, resulting in augmented NO generation by endothelial cells and subsequent vasodilation (Siamwala et al. 2013).

One biomarker of inflammation is tumor necrosis factor alpha (TNF-α). TNF-α is elevated in obesity, linked to insulin resistance and stimulates the development of type 2 diabetes and cardiovascular disease as observed in diabetic vascular complications (Alexandraki et al. 2006). TNF-α induces the expression of adhesion molecules and endothelial dysfunction, which stimulates development of atherosclerosis. The expression of endothelial adhesion molecules promotes the attachment of leukocytes and subsequently leads to atherogenesis. Simultaneously, endothelial cells stimulated by TNF-α induce inflammation and stimulate apoptosis (Wu et al. 2009). On the other hand, EGCG inhibits TNF-α-induced plasminogen activator inhibitor-1 (PAI-1) production via ERK1/2 phosphorylation in endothelial cells (Cao et al. 2013). Furthermore, EGCG attenuates TNF-α-induced production of monocyte chemotactic protein-1 (MCP1) and reduces Akt phosphorylation (Ahn et al. 2008). EGCG also inhibits phorbol 12-myristate 13-acetate (PMA)-induced MCP1 expression and THP-1 cell migration by suppressing p38 MAPK and NF-kappa B activation.

A recent study reported that EGCG reduces palmitate-induced accumulation of lipid droplets during autophagy in endothelial cells (Kim et al. 2013). Other reports indicated that the activation of p38 and ERK1/2 was associated with expression of similar endothelial adhesion molecules and the induction of apoptosis (Chae et al. 2007; Das et al. 2010). However, the mechanisms underlying the capacity of green tea components to inhibit endothelial adhesion molecule expression and apoptosis during atherosclerosis remain unclear. The purpose of this study was to elucidate the protective effects of the green tea components EGCG and L-theanine against TNF-α-induced stimulation of endothelial cells, expression of adhesion molecules and apoptosis, which is associated with autophagy.

Materials and methods

Materials

EGCG, L-theanine and resveratrol were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Fetal bovine serum (FBS), TRizol reagent, DNase and Superscript III were purchased from Life Technologies Japan, Inc. (Tokyo, Japan). Bradford reagent was obtained from Bio-Rad (Richmond, CA, USA). LC3A and LC3B antibodies were purchased from Cell Signaling Technology Japan K.K. (Tokyo, Japan) and a VCAM1 antibody was purchased from Abcam, Inc. (Cambridge, MA, USA). β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Human TNF-α was obtained from Roche Applied Science (Mannheim, Germany). The caspase 3/7 activity kit was purchased from Life Technologies Japan Inc. U937 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). 3-Methyladenine (3-MA) was obtained from Tocris Biosciences (Minneapolis, MN) and dissolved in dimethyl sulfoxide (DMSO). We confirmed that the DMSO vehicle alone did not affect our results. All other reagents were purchased from Sigma-Aldrich unless otherwise indicated. Stock EGCG, L-theanine and resveratrol solutions (100 mM) were prepared in DMSO and stored at −20 °C.

Cell culture

The human endothelial cell line ISO-HAS was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). ISO-HAS cells were cultured in 50% Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and conditioned medium from the angiosarcoma cell line ISO-1 (1:1, DMEM/ISO-1) (Masuzawa et al. 1999). The ISO-HAS cell line was established from tumor tissue from a human hemangiosarcoma. However, ISO-HAS cells were found to constitutively express the von Willebrand factor (vWF), Flt-1, KDR, CD31 and vascular endothelial growth factor (VEGF) (Amo et al. 2001; Unger et al. 2002). The cells were seeded at a ratio of 1:1 in culture flasks (75 cm^2, Becton Dickinson, Bedford, MA) and grown in DMEM/ISO-1 at 37 °C under 5% CO_2, and in a humidified atmosphere. The medium was periodically renewed until the cells reached 80–90% confluence, at which point they were treated with 0.25% trypsin (Sigma). The human monocyte U937 cells were cultured in RPMI-1640 supplemented with 10% (w/w) heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. U937 cells were grown in suspension cultures and subcultured twice weekly in culture flasks.

Adhesion of endothelial and U937 cells

ISO-HAS cells were cultured for 24 h without or with TNF-α (10 ng/ml) and/or EGCG (10 and 30 μM) or L-theanine (10 and 30 μM). U937 cells were pretreated with 1 μM Cell Tracker Green (Life Technologies Japan) for 30 min at 37 °C and subsequently allowed to interact with ISO-HAS and labeled U937 (1×10^6 cells/ml) cells for three h at 37 °C. Unbound U937 cells were removed by gently washing twice with PBS, and adherent labeled-U937 cells were photographed with an BZ-8000 fluorescent microscope (Keyence, Osaka, Japan). The total number of labeled-U937 cells bound to endothelial cells was determined using fluorescence intensity (FI) values and cell numbers. Image analysis was carried out with the BZ-8100 Dynamic Cell Count image analysis program (Keyence). For quantification of results, fluorescence intensity was measured under the same exposure conditions in the same area as that used with the BZ-8000 fluorescent microscope (Keyence).