**Regular Article**

**Inhibitory Effect of (−)-Epigallocatechin-3-O-gallate on Octanoylated Ghrelin Levels in Vitro and in Vivo**

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Ghrelin is an orexigenic peptide hormone produced in the stomach. The major active form is octanoylated ghrelin, which is modified with an n-octanoic acid at the serine-3 residue. Inhibition of octanoylated ghrelin production is useful for the prevention and improvement of obesity. We previously developed a cell-based assay system employing a ghrelin-expressing cell line, AGS-GHRL8, and found various compounds that decreased octanoylated ghrelin levels using this system. (−)-Epigallocatechin-3-O-gallate (EGCG) is a bioactive catechin in green tea and reportedly has an anti-obesity effect; however, it remains unclear whether EGCG inhibits octanoylated ghrelin production. Therefore, in this study, we investigated the effect of EGCG on octanoylated ghrelin levels in AGS-GHRL8 cells and C57BL/6J mice. EGCG significantly reduced the octanoylated ghrelin level in AGS-GHRL8 cells. In mice, three days of treatment with TEAVIGO\(^\text{®}\), which contains 97.69% EGCG, lowered the plasma octanoylated ghrelin level by 40% from that in control mice. In addition, TEAVIGO\(^\text{®}\) reduced the mRNA expression of ghrelin and prohormone convertase 1/3, an enzyme responsible for the processing of proghrelin to mature ghrelin, in the mouse stomach, suggesting that the reduced expression of these genes may contribute to the inhibition of octanoylated ghrelin production. These results suggest a decrease in the octanoylated ghrelin level to be involved in the anti-obesity effect of EGCG, which thus has potential for the development of anti-obesity agents with ghrelin-lowering effect.

**Key words** (−)-epigallocatechin-3-O-gallate (EGCG); octanoylated ghrelin; AGS-GHRL8 cell

Ghrelin is a 28-amino-acid peptide identified in 1999 as an endogenous ligand for the growth hormone secretagogue receptor\(^1\) and induces body weight gain by promoting food intake.\(^2\) It circulates mainly in two forms, octanoylated ghrelin and des-acyl ghrelin.\(^3\) Octanoylated ghrelin, having an octanoyl modification at the serine-3 residue, exerts an orexigenic effect, while des-acyl ghrelin, without acyl modification, does not.\(^4\) Therefore, a reduction in the octanoylated ghrelin level would be effective for the prevention and improvement of obesity. Ghrelin is synthesized in gastric endocrine cells (X/A-like cells).\(^5\) Ghrelin mRNA is translated into a precursor protein, Proghrelin, which is cleaved by ghrelin O-acyltransferase (GOAT).\(^6\) Proghrelin is subjected to the cleavage of several enzymes, including furin, PC1/3, and PC2.\(^6,10,11\) Mature octanoylated ghrelin is stored in granules and secreted into circulation by exocytosis.\(^5\)

We previously established a ghrelin-expressing cell line, AGS-GHRL8, by transfecting the ghrelin gene into human AGS gastric carcinoma cells.\(^12\) AGS-GHRL8 cells express both GOAT and furin and produce octanoylated ghrelin in the presence of octanoic acid. We developed a cell-based assay system employing AGS-GHRL8 to explore inhibitors of octanoylated ghrelin production.\(^12\) Using this assay system, we identified various fatty acids, including heptanoic acid, stearic acid, linoleic acid, α-linolenic acid and oleic acid and some triterpenes, such as asiatic acid, corosolic acid, glikycrhetic acid, oleanolic acid, and ursolic acid, which suppress the octanoylated ghrelin level in culture medium of AGS-GHRL8 cells.\(^12–14\)

Tea is one of the most consumed beverages in the world and has been reported to have cancer- and heart disease-preventive effects.\(^15\) Tea is rich in polyphenols, most of which are catechins. Green and black tea, the major commercial types of tea, contain 30–40% and 3–10% catechins, respectively.\(^16,17\) Tea rich in catechins has been reported to have an anti-obesity effect.\(^18,19\) Upregulation of energy expenditure and the reduction in glucose absorption by inhibition of α-glucosidase activity were reported to be involved in the anti-obesity mechanisms of tea catechins.\(^20,21\) (−)-Epigallocatechin-3-O-gallate (EGCG) (Fig. 1) is the major catechin in tea,\(^16\) and supplements containing EGCG, such as TEAVIGO\(^\text{®}\) (>94% EGCG) and Sunphenon\(^\text{®}\) EGCG-OP (>94% EGCG) are commercially available. Several studies have suggested that EGCG reduces food intake\(^22\) and has an anti-obesity effect.\(^23,24\) An elevation of thermogenesis and a decrease in lipid absorption are reportedly involved in the anti-obesity effect of EGCG.\(^25,26\) However, the effect of EGCG on octanoylated ghrelin production has not yet been clarified.

In this study, we examined the effects of EGCG on octanoylated ghrelin levels in vitro and in vivo. Furthermore, we investigated the expression of ghrelin and enzymes involved in octanoylated ghrelin production in AGS-GHRL8 cells and C57BL/6J mice.

**MATERIALS AND METHODS**

**Reagents and Materials** EGCG, octanoic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human and rat octanoylated ghrelin enzyme-linked immunosorbent assay
EGCG, was added to each well. After 24 h of culture, the medium was collected in a microtube, and 1/10 volume of 1 mol/L HCl was mixed into each sample. The remainder of cells in each well were washed with PBS twice and harvested using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid solution. The cell number was determined with an EVE Automatic cell counter (Ar Brown, Tokyo, Japan). The octanoylated ghrelin concentration in the culture medium was measured with the human octanoylated ghrelin ELISA kit, and was adjusted for the number of the cells in each well.

Evaluation of the Effect of EGCG on the Plasma Level of Octanoylated Ghrelin in Mice Six-week-old male C57BL/6J mice (SLC, Hamamatsu, Japan) were maintained at room temperature under a 12-h light–dark cycle (light on/off: 7:00/19:00). The mice were fed a normal diet (CE-2; CLEA Japan, Tokyo) and water ad libitum. After a 1-week habituation, the mice were assigned to two body weight-matched groups. The mice were given TEAVIGO® dissolved in ultrapure water (100 mg/kg, orally) or vehicle (ultrapure water) eight times every 8 h for three days. Blood and stomach samples were collected from each mouse under anesthesia at 19:00 after 6 h of fasting. Plasma was collected by centrifugation of the blood samples at 2000 × g for 5 min, and was mixed with 1/10 volume of 1 mol/L HCl and stored at −80°C until analysis of the octanoylated ghrelin concentration. Stomach tissue was stored in RNA later until total RNA extraction. Plasma octanoylated ghrelin concentrations were measured using the rat octanoylated ghrelin ELISA kit. This experiment was approved by the Research Ethics Committee of the Faculty of Pharmaceutical Sciences (Nagasaki International University) (No. 123-2) and was conducted in accordance with Ethical Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences (Nagasaki International University).

Quantitative RT-PCR Quantitative (q)RT-PCR was performed as described previously. Total RNA was extracted from cells and mouse stomach [2, 13, 14] using TRIzol (Life Technologies, Carlsbad, CA, U.S.A.) or the CFX Connect Real-Time System (Bio-Rad, Tokyo, Japan). A reaction solution consisting of 10 µL of diluted cDNA was used. The reaction mixtures were subjected to an initial denaturation at 95°C for 20 s, followed by 40 cycles of amplification at 95°C (1 s) for denaturation, and 60°C (20 s) for annealing and extension. The sequences of the primers used for qRT-PCR were as follows: human GOAT, sense: 5′-ACA GCT CGA TGG CTC CGA CGC-3′ and antisense: 5′-AGCTT CCACCATCACCGGCC-3′, human furin, sense: 5′-GAA GTG CAC AGA ATG-3′ and antisense: 5′-GCC ATG CTG CTG-3′, mouse PC2, sense: 5′-ACC TGG TTT CAC TAT GC-3′ and antisense: 5′-GCC ATG CTG CTG-3′, mouse GHRL8, sense: 5′-ACC TGG TTT CAC TAT GC-3′ and antisense: 5′-GCC ATG CTG CTG-3′.
18S ribosomal RNA, sense: 5'-GTA ACC CGT TGA ACC CCA TT-3' and antisense: 5'-CCA TCC AAT CGG TAG TAG CG-3'. Transcript levels were estimated from the respective standard curves and were normalized to the amount of 18S ribosomal RNA.

Statistical Analysis Values are given as mean±standard deviation (S.D.). Differences between groups were analyzed by a two-sample t-test or Tukey’s test for multiple comparisons. Differences were considered significant at p<0.05.

RESULTS

Effects of EGCG on the Octanoylated Ghrelin Level and mRNA Expression of GOAT and Furin in AGS-GHRL8 Cells We first assessed the viability of AGS-GHRL8 cells upon treatment with EGCG at concentrations ranging from 0 to 100 µmol/L by MTT assay to select test concentrations. As there was no significant difference between control and EGCG-treated cells (Fig. 2A), subsequent experiments were carried out using 50 and 100 µmol/L of EGCG, which were the nontoxic concentrations. EGCG at 100 µmol/L significantly decreased the octanoylated ghrelin concentration in the culture medium by 31% as compared to the control, while 50 µmol/L of EGCG induced no significant change (Fig. 2B).

In addition, we evaluated the effects of EGCG on GOAT and furin mRNA expression by qRT-PCR. GOAT and furin mRNA levels of EGCG-treated cells were not significantly different from those of the control (Fig. 2C).

Effects of EGCG on the Plasma Octanoylated Ghrelin Level and Expression of Ghrelin, GOAT, Furin, PC1/3, and PC2 mRNA in Mice Next, we investigated the effect of EGCG on octanoylated ghrelin production in vivo. Oral administration of TEAVIGO®, which contains 97.69% EGCG, significantly reduced the plasma octanoylated ghrelin concentration in mice (Fig. 3A). There were no significant differences in body weight gain between the control and TEAVIGO®-treated mice (data not shown).

We also compared the mRNA expression levels of ghrelin, GOAT, furin, PC1/3 and PC2 in stomachs of TEAVIGO®-treated mice to those in control mouse stomachs. The mRNA expression of ghrelin and PC1/3 was significantly downregulated in TEAVIGO®-treated mice, while expression of GOAT, furin, and PC2 did not differ between the two groups (Fig. 3B).

Effects of Hydrolysates of EGCG on the Octanoylated Ghrelin Level in AGS-GHRL8 Cells It has been reported that EGCG is converted to (−)-epigallocatechin (EGC) by esterase in the oral cavity in humans. Furthermore, it has been reported that propyl gallate, an ester of gallic acid, is hydrolyzed by esterase in the body. From these reports, we hypothesized that EGCG is converted to EGC or gallic acid in the body (Fig. 4A), and we examined the effects of EGC and gallic acid on the octanoylated ghrelin level in culture medium of AGS-GHRL8 cells. The maximum nontoxic concentrations of EGC and gallic acid as determined by MTT assay were 100 and 50 µmol/L, respectively (data not shown).
Fig. 3. Effect of EGCG on the Plasma Octanoylated Ghrelin Level in C57BL/6J Mice
Mice were administered TEAVIGO®, a green tea extract with 97.69% EGCG, for three days. (A) Plasma octanoylated ghrelin concentration in C57BL/6J mice treated with TEAVIGO®. Data are presented as the mean±S.D. (n=10). *p<0.05 vs. control group. (B) mRNA levels of ghrelin, GOAT, furin, PC1/3, and PC2 in stomachs of mice treated with TEAVIGO® relative to those in control mouse stomachs. Data are expressed as the mean±S.D. (n=8–10). *p<0.05 vs. control group.

Fig. 4. Effects of (−)-Epigallocatechin (EGC) and Gallic Acid on the Octanoylated Ghrelin Level in AGS-GHRL8 Cells
(A) Structures of EGC and gallic acid. (B) Concentrations of octanoylated ghrelin in culture medium of EGC- or gallic acid-treated AGS-GHRL8 cells relative to those in control cell medium. Octanoic acid was added at 100µmol/L in all wells. Data are expressed as the mean±S.D. (n=6).
Significant changes in the octanoylated ghrelin concentration in the culture medium of AGS-GHRL8 cells treated with EGC (50 and 100 μmol/L) or gallic acid (25 and 50 μmol/L) were not observed (Fig. 4B).

DISCUSSION

This study demonstrated that EGCG lowered the octanoylated ghrelin level in a ghrelin-expressing cell line, AGS-GHRL8, as well as the plasma level of octanoylated ghrelin in C57BL/6J mice. In our in vivo study, three-day EGCG treatment decreased the plasma octanoylated ghrelin level, but did not affect body weight gain. In previous studies showing the weight loss effect of EGCG, the administration period was several weeks. The administration period in this study may be too short to observe body weight loss. A decrease in plasma octanoylated ghrelin reportedly reduces food intake and body weight. Therefore, EGCG may have therapeutic potential for obesity through lowering ghrelin.

EGCG is known to affect the mRNA expression of various proteins, such as vascular endothelial growth factor, medium-chain acyl CoA decarboxylase, and peroxisome proliferator-responsive element. We evaluated the effects of EGCG on mRNA expression of ghrelin, GOAT, furin, PC1/3, and PC2, which are involved in the octanoylated ghrelin production process, to elucidate the mode of action of EGCG. Ghrelin and PC1/3 mRNA levels in the mouse stomach were downregulated by EGCG treatment, suggesting that the reduction in ghrelin and PC1/3 mRNA expression levels may be involved in the decrease in plasma octanoylated ghrelin in mice. The mRNA expression of GOAT and furin did not change in mice and AGS-GHRL8 cells. As AGS-GHRL8 cells constitutively express ghrelin, and mRNA expression of PC1/3 and PC2 has not been identified, this cell line is unsuitable for investigation of the effects of EGCG on ghrelin, PC1/3, and PC2 gene expression.

Being an ester of EGC and gallic acid, EGCG can be hydrolyzed to EGC and gallic acid by blood esterase, similar to its hydrolysis by salivary esterase. When we evaluated the effects of EGC and gallic acid on the octanoylated ghrelin level in AGS-GHRL8 cells, both molecules did not show an effect. These results indicate that it is highly probable that EGCG itself is the active molecule in lowering octanoylated ghrelin. Although EGCG and EGC are tea catechins with a common chemical structure, EGCG seems to have some unique activities.

Octanoylated ghrelin is synthesized in X/A-like cells of the stomach and is secreted from these cells by exocytosis. EGCG reportedly suppresses the exocytosis of histamine through the 67-kDa laminin receptor (67LR). Galloylated catechins such as EGCG bind to 67LR, but non-galloylated catechins such as EGC and epicatechin do not. In this study, EGCG, but not EGC, decreased the octanoylated ghrelin level in the culture medium of AGS-GHRL8 cells. Since AGS-GHRL8 cell line was established from human gastric carcinoma AGS cells, in which 67LR is expressed, the difference in the inhibitory effect of EGCG and EGC on octanoylated ghrelin production and secretion may be due to a difference in their binding abilities to 67LR. Only limited information is available regarding the relationship of octanoylated ghrelin and 67LR, and thus, further studies are needed to elucidate their relationship. TEAVIGO® (Lot:504091) contains 1.57% (->)-epicatechin-3-O-gallate and 0.50% (->)-gallolechin-3-O-gallate. Since galloylated catechins bind to 67LR, these catechins may exert inhibitory effect on octanoylated ghrelin production. However, the concentration of these two catechins were very low compared with that of EGCG. Therefore, the decrease in the plasma level of octanoylated ghrelin in mice administered TEAVIGO® could be due to the action of EGCG.

In this study, we demonstrated that EGCG suppressed the levels of octanoylated ghrelin in vitro and in vivo. The results suggest that the decrease in octanoylated ghrelin may be involved in the anti-obesity effects of EGCG, the most abundant catechin in tea. As previous studies have indicated the safety of EGCG administration, EGCG has potential as a safe and effective anti-obesity agent through lowering ghrelin.

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Conflict of Interest The authors declare no conflict of interest.

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