Epigallocatechin Gallate (EGCG) Stimulates Autophagy in Vascular Endothelial Cells

A POTENTIAL ROLE FOR REDUCING LIPID ACCUMULATION**‡

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Background: Green tea polyphenol (EGCG) has beneficial effects on cardiovascular dysfunction.

Results: EGCG stimulates autophagy through a CaMKKβ-mediated mechanism, which contributes to degradation of lipid droplets.

Conclusion: Regulation of autophagic flux by EGCG plays a role in intracellular lipid accumulation.

Significance: Findings show a novel mechanism for beneficial effects of EGCG in cardiovascular complications.

Epigallocatechin gallate (EGCG) is a major polyphenol in green tea that has beneficial effects in the prevention of cardiovascular disease. Autophagy is a cellular process that protects cells from stressful conditions. To determine whether the beneficial effect of EGCG is mediated by a mechanism involving autophagy, the roles of the EGCG-stimulated autophagy in the context of ectopic lipid accumulation were investigated. Treatment with EGCG increased formation of LC3-II and autophagosomes in primary bovine aortic endothelial cells (BAEC). Activation of calmodulin-dependent protein kinase kinase β was required for EGCG-induced LC3-II formation, as evidenced by the fact that EGCG-induced LC3-II formation was significantly impaired by knockdown of calmodulin-dependent protein kinase kinase β. This effect is most likely due to cytosolic Ca2+ load. To determine whether EGCG affects palmitate-induced lipid accumulation, the effects of EGCG on autophagic flux and co-localization of lipid droplets and autophagolysosomes were examined. EGCG normalized the palmitate-induced impairment of autophagic flux. Accumulation of lipid droplets by palmitate was markedly reduced by EGCG. Blocking autophagosomal degradation opposed the effect of EGCG in ectopic lipid accumulation, suggesting the action of EGCG is through autophagosomal degradation. The mechanism for this could be due to the increased co-localization of lipid droplets and autophagolysosomes. Co-localization of lipid droplets with LC3 and lysosome was dramatically increased when the cells were treated with EGCG and palmitate compared with the cells treated with palmitate alone. Collectively, these findings suggest that EGCG regulates ectopic lipid accumulation through a facilitated autophagic flux and further imply that EGCG may be a potential therapeutic reagent to prevent cardiovascular complications.

Ectopic accumulation of lipids, including neutral lipid and cholesterol esters, contributes to inflammatory status and endoplasmic reticulum (ER) stress in vascular endothelium that is associated with endothelial dysfunction and atherosclerosis (1). Degradation of lipid droplets by stimulation of autophagy (lipophagy) reduces ER stress and inflammation (2). Autophagy is a catabolic process that plays pivotal roles in metabolism, cell death, and differentiation (3, 4). An excess amount of lipids, aggregated proteins, and organelles is degraded through the autophagic process, which is one of the protective mechanisms used to remove unused cellular materials (5, 6). Macroautophagy (hereafter autophagy) occurs through a series of events forming membrane-like structure, compartmentalization, and fusion of vesicles that generate autophagolysosome (5). Impairment of the lysosomal degradation process causes reduced autophagic flux leading to serious disorders in cardiovascular and metabolic tissues (7–9).

An 11-year follow-up study shows that green tea consumption is associated with reduced mortality due to cardiovascular diseases but not with mortality due to cancer (10). We and others have shown that the most abundant green tea polyphe-
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ol, epigallocatechin-3-gallate (EGCG), has beneficial health effects in various pathophysiological conditions, including insulin resistance, endothelial dysfunction, and ischemia-reperfusion injuries (11–13). One of the molecular mechanisms for EGCG-mediated protective effects is through activation of adenosine monophosphate-activated protein kinase (AMPK) (14, 15). However, the molecular mechanisms for linking EGCG-stimulated AMPK and autophagy with regard to lipid metabolism are not known. Furthermore, although polyphenols, including EGCG and resveratrol, have effects on lipolysis, it is not known whether the lipolysis is associated with autophagy (16, 17).

In this study, we investigated the mechanism of EGCG-induced autophagy and its role in accumulation of intracellular lipid accumulation. Here, we show that EGCG-stimulated autophagy is (at least in part) through a CaMKKβ/AMPK-mediated mechanism and has a role in degradation of lipid droplets in vascular endothelial cells. Our data demonstrate a novel mechanism for polyphenols to regulate lipid metabolism in vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—Anti-LC3 (4599), anti-ATG5 (8540), anti-pAMPK (2531), anti-AMPK (2532), anti-pAkt (9271), anti-pULK1 (5869), anti-ULK1 (4773), anti-pmTOR (2971), and anti-mTOR (2972) antibodies were obtained from Cell Signaling Technology. Anti-CaMKKβ (sc-50341) and anti-LAMP-1 (sc-17768) antibodies were obtained from Santa Cruz Biotechnology. EGCG (Sigma, E4143) and anti-β-actin (Sigma, A5316) antibody were obtained from Sigma. Anti-SQSTM1 (p62) was purchased from BD Biosciences (610832). Dicer siRNA for bovine ATG5 and CaMKKβ and scrambled dicer siRNA were purchased from Integrated DNA Technologies. 3-Methylaniline (3977), PD98059 (1213), SB203580 (1202), compound C (3093), and STO-609 (1551) were obtained from Tocris Biosciences ( Minnesota, MN). Some of the inhibitors were dissolved in dimethyl sulfoxide (DMSO), and we confirmed that the vehicle alone did not affect our results.

Cell Culture and Transfection—Bovine aortic endothelial cells (BAEC) in primary culture were obtained from Cell Applications (San Diego) and maintained in F-12K media containing 5% fetal bovine serum (FBS), endothelial cell growth supplement (15 μmol/ml, CB40006B, BD Biosciences), heparin sulfate (50 μg/ml, Sigma, H3393), penicillin (100 units/ml), and streptomycin (100 μg/ml). All experiments were conducted on BAEC between three and six passages. BAEC were transfected with dicersiRNA using reagents (Polyplus Transfection, 409-50) according to the manufacturer’s instructions. One day after transfection, cells were serum-starved for 2 h and then treated with EGCG or inhibitors as indicated in the legends to figures. The sequences are as follows: dicer siRNA for bovine agt, 5′-rArArUrCrUrCrUrCrUrGrUrCrUrArArGrUrU-3′ and 5′-rCrUrUrGrArUrArGrArGrArCrArGrArGrArGrArGrArGrArGrATT-3′; bovine CaMKKβ, 5′-rGrUrUrCrUrGrUrGrUrGrGrUrGrCrArUrCrUrGrGrGrUrGr-GrU-3′ and 5′-rCrArGrArArUrCrCrArCrUrCrUrCrUrCrArArArAGC-3′; and dsi scrambled RNA, 5′-rArUrAr-

Calcium Measurements—The intracellular Ca2+ levels in BAEC were recorded using the Ca2+ indicator fluo-3 (Invitrogen, F-14218) and a modification of previously described procedures (18, 19). Cell suspension was applied onto polyethyleneimine (1 mg/ml)-coated coverslips. Cells were allowed to adhere for 1 h, upon which floating cells were washed away.
Coverslips containing adhered cells were placed overnight in culturing medium in a 5% CO₂, 95% air atmosphere incubator at 37 °C. The following day, after 2 h of serum starvation at 37 °C, BAEC were loaded with the acetoxymethyl (AM) ester of fluo-3 (10 μM) for 20 min at room temperature (20–24 °C) in external solution containing (in mM) the following: 140 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 5 glucose, and 10 HEPES (pH 7.4); dispersion of the ester was aided by 0.025% Pluronic F-127 (Invitrogen, P3000MP). After washing, the indicator was permitted to de-esterify for 20 min in BAEC at room temperature in external solution. Coverslips containing fluo-3-loaded BAEC were then mounted onto a recording chamber and visualized. Images were acquired every 5 s, the first 10 s being used as a base line to establish \( F_0 \) (see below). After image 10, we bath applied EGCG (10 μM, 200 s) to test its effects on intracellular Ca²⁺ levels. Ca²⁺ responsiveness was confirmed in cells at the end of each experiment by using the Ca²⁺ ionophore 4-bromo-A23187 (10 μM, 200 s, B1494, Invitrogen) as described previously (20).

To assess contribution of the ER Ca²⁺ store to EGCG-induced Ca²⁺ dynamics in BAEC, we employed a commonly used procedure described elsewhere (21). Briefly, BAEC were preincubated in external solution supplemented with cyclopiazonic acid (CPA, 20 μM, 30 min; Sigma, C1530), a blocker of ER Ca²⁺-ATPase. After allowing the ER store to deplete for 30 min, we bath applied EGCG in the presence of CPA.
All experiments were done at room temperature. We used an inverted microscope (TE 300; Nikon, Melville, NY) equipped with wide field epifluorescence.

For visualization of fluo-3, we used a standard fluorescein/FTC filter set (Chroma, Rockingham, VT). Images were captured through a ×60 PlanApo oil-immersion objective (numerical aperture (NA), 1.4; Nikon) using a CoolSNAP-HQ-cooled charge-coupled device camera (Roper Scientific, Tucson, AZ) driven by V++ imaging software (Digital Optics, Auckland, New Zealand). For time-lapse image acquisition, a camera and an electronic shutter (Vincent Associates, Rochester, NY) inserted in the excitation pathway were controlled by software. A xenon arc lamp (100 watts) was used as a light source. All images shown in figures and supplemental movies represent raw data with their pixel intensities within the camera’s (CoolSNAP-HQ) dynamic range (0–4095). In analysis, all imaging data were background subtracted using regions of the coverslip field containing no cells. Data are expressed as dF/F0 (%), where dF represents the change of fluorescence, and F0 represents the base-line fluorescence of cells (10 consecutive images). Ca2+ transients from background subtracted fluo-3 emission traces were declared Ca2+ peaks if over two consecutive images their dF values exceeded the F0 ± S.D. Oscillatory events were counted for each cell, and their peaks (amplitudes) dF/F0 were determined. Cumulative Ca2+ responses were calculated by summing dF/F0 for individual time points during the entire EGCG challenge of BAECs.

All data on Ca2+ dynamics fulfill normality as established using the Shapiro-Wilk test. The effects of CPA on EGCG-induced Ca2+ dynamics (number of oscillatory events, peak and cumulative dF/F0) were tested using t tests. Data are expressed as means ± S.E.

RT-PCR—The cells were treated as described in the figure legends. Total RNA was prepared by using TRIzol (Invitrogen, 15596018) according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis by using the Omniscript RT kit (Qiagen, 205113). Then the cDNA was subjected to semi-quantitative PCR analysis by using Hot Star Taq Master Mix kit (Qiagen, 203445). PCR product was visualized with fluorescent dye (Helix Technologies, HDS001), and the image was analyzed and quantified by Image analyzer (Vision Works LS) and UVP. The primers for CaMKKβ are forward, TGAAGACCCAGGCCGTCTTTACTT, and reverse, TCACACCAAAAGTCCGGCAGTCTTG; and for β-actin are forward, CTGGCACCCAGCACAATGAAG, and reverse, TGAAGCATTGCGGTGGACG.

RESULTS

EGCG Stimulates Autophagy—Because formation of LC3-II by cleavage and lipidation is an indication of autophagy (22–24), we examined whether EGCG stimulates LC3-II formation. Treatment of BAEC with EGCG increased LC3-II formation in a time-dependent (Fig. 1, A and B) and dose-dependent (Fig. 1, C and D) manner. To confirm that EGCG-stimulated LC3-II formation is through an autophagy-dependent mechanism, we transiently transfected BAEC with siRNA for atg5 and then treated BAEC without or with EGCG. Knockdown of atg5 (one of the genes required for autophagy, 40% reduction in ATG5 protein expression) reduced EGCG-stimulated LC3-II formation (Fig. 1, E and F). The data suggest that EGCG-stimulated LC3-II formation is through an autophagy-dependent mechanism. Next, we also observed the autophagosome formation by electron microscopy after treating the BAEC without or with EGCG (10 μM) (Fig. 2). EGCG stimulated formation of autophagolysosome-like structure and made compartments that have less dense areas compared with the normal cellular density (white triangles) and autophagosomes (Fig. 2A, solid line arrow). Phagophore and double membrane structures indi-
cate that active autophagy occurs with EGCG treatment (Fig. 2B). The autophagosome area (Fig. 2C) and autophagolysosomal (Fig. 2D) area were increased 3- and 7-fold, respectively, in the EGCG-treated cells when compared with untreated cells. The rates of autophagosome formation and degradation are steady state in normal conditions, which is altered in various stressful conditions (25). We examined whether EGCG stimulates autophagic flux by comparing accumulation of LC3-II with and without inhibition of lysosomal degradation. We blocked lysosomal degradation by using ammonium chloride/leupeptin (NH4Cl/Leu) as reported previously (26). Treatment with NH4Cl/Leu alone causes accumulation of LC3-II, and treatment with EGCG and NH4Cl/Leu further enhanced the accumulation of LC3-II (Fig. 3, A and B). The subtracted values (the difference in the amount of LC3-II without blocking lysosomal degradation from the value with lysosomal degradation) indicate that EGCG stimulates autophagic flux (Fig. 3C). Sequestosome 1 (SQSTM1, p62), a ubiquitin-binding protein, is involved in autophagy and is known as an indication of autophagic degradation (27–29). We examined whether EGCG facilitates degradation of SQSTM1. Treatment of EGCG significantly decreased the SQSTM1 level (Fig. 3, D and E). This suggests that EGCG enhances autophagic degradation.

**EGCG-stimulated LC3-II Formation Is through a CaMKKβ/AMPK-mediated Mechanism**—We previously reported that EGCG stimulates AMPK (30). Because AMPK directly phosphorylates uncoordinated-51-like kinase (ATG1/ULK1) (31, 32), we examined whether EGCG stimulates phosphorylation of AMPK and ULK1. Treatment of BAEC with EGCG stimulated the phosphorylation of AMPK and ULK1 in a time-dependent manner (Fig. 4A). To identify signal transduction pathways for EGCG-stimulated autophagy, we treated BAEC with various inhibitors. EGCG-stimulated LC3-II formation was inhibited by 3-methyladenine (PI3K inhibitor), compound C (AMPK inhibitor), and STO-609 (CaMKKβ inhibitor) but not by PD98059 (MEK inhibitor) and SB203580 (p38 MAPK inhibitor) (Fig. 4, B and C). We previously demonstrated that EGCG stimulates H2O2 production, which is known to stimulate CaMKKβ and AMPK in vascular endothelial cells (33). Thus, we examined whether H2O2 is involved in LC3-II formation.

**FIGURE 3. EGCG enhances autophagic flux.** A and B, BAEC were treated without or with EGCG (10 μM, 4 h). The lysosomal inhibitor (NH4Cl (20 mM), Leu (200 μM)) was treated 1 h prior to cell harvest, and cell lysate was collected and analyzed by immunoblotting for LC3-II formation. Three independent experiments were performed, and the density of LC3-II/LC3-I was quantified. Data are mean ± S.E. (***, p < 0.001). C, differences between the absence and presence of NH4Cl/Leu were calculated for the indication of autophagic flux. Three independent experiments were quantified and calculated. Data are mean ± S.E. (*, p < 0.05). D and E, BAEC were treated without or with EGCG (10 μM) for the indicated time points. Cell lysate was harvested and analyzed by immunoblot with anti-SQSTM1(p62) antibody. SQSTM1 was significantly degraded by treatment with EGCG, which indicates autophagic degradation was enhanced. Three independent experiments were performed, and data are mean ± S.E. (***, p < 0.001). NT, not treated.
However, pretreatment of cells with N-acetylcysteine, an antioxidant, was not able to inhibit the EGCG-stimulated LC3-II formation (Fig. 4, D and E). In contrast, the same amount of N-acetylcysteine was able to inhibit EGCG-stimulated phosphorylation of Akt (Fig. 4, F and G). Inhibition of CaMKKβ by STO-609 inhibited EGCG-stimulated phosphorylation of AMPK and ULK1 (Fig. 5, A and B). mTOR plays an important role in autophagy, and inhibition of mTOR stimulates autophagy in response to various stimuli (34, 35). We examined whether EGCG inhibits phosphorylation of mTOR. Surprisingly, EGCG did not inhibit the phosphorylation of mTOR, and pretreatment with STO-609 did not affect the EGCG-stimulated phosphorylation of mTOR (Fig. 5C). These data suggest that the EGCG-stimulated autophagy is independent of mTOR.
activity. To confirm that CaMKKβ is involved in EGCG-stimulated LC3-II formation, we transiently transfected BAEC with siRNA for CaMKKβ, and we then treated with EGCG. Knockdown of CaMKKβ by siRNA was able to reduce the mRNA expression of CaMKKβ by 48%. Reduction of CaMKKβ inhibited EGCG-stimulated LC3-II formation (Fig. 5D). Thus, the data suggest that EGCG stimulates autophagy through a CaMKKβ-mediated mechanism.

**EGCG Stimulates Cytosolic Ca²⁺ Levels That Are Required for LC3-II Formation**—Because CaMKKβ requires intracellular calcium signaling to be activated (36), we evaluated if BAEC have the ability to increase cytosolic Ca²⁺ (Ca²⁺ cyt) in response to EGCG. Serum-starved BAEC were loaded with the Ca²⁺ dye fluo-3 to monitor their Ca²⁺ cyt levels. Bath applied EGCG (10 μM, 200 s) caused an increase in Ca²⁺ cyt levels, which displayed an oscillatory pattern (n = 44) (supplemental Movie 1). It has been established that in other cells, e.g. astrocytes, this oscillatory pattern resembles calcium release from internal stores, mainly ER (19, 37). To test whether internal Ca²⁺ stores, in particular the ER store, supply Ca²⁺ during EGCG stimulation of BAEC, we pretreated serum-starved BAEC with CPA (20 μM, 30 min), a widely used blocker of ER Ca²⁺-ATPase. During the course of this pretreatment with CPA, the depletion of the ER store in the presence of extracellular Ca²⁺ was evident as a slow increase in Ca²⁺ cyt levels (n = 36; ΔF₀/F₀ = 47 ± 3%; p < 0.01, paired t test), which reached a new baseline (Fig. 6, compare images a in A and B). Because we were interested in the ability of BAEC to handle Ca²⁺ cyt once the ER store had been depleted, we used Ca²⁺ cyt levels after the CPA pretreatment as a baseline (F₀) for further analysis. Indeed, CPA was kept throughout the remainder of the experimental paradigm. Hence, bath application of EGCG to CPA-treated cells (n = 36) caused attenuated Ca²⁺ cyt dynamics (Fig. 6B and supplemental Movie 2); the average number of oscillatory events was reduced when compared...
with those recorded from the control cells (n = 44) exposed to EGCG in the absence of CPA (Fig. 6C; 3.3 ± 0.6 and 5.7 ± 0.9, respectively). Furthermore, peaks/amplitudes of oscillatory events were decreased in CPA-treated cells in comparison with control cells (peak dF/F0 = 24 ± 7 and 55 ± 16%, respectively) (Fig. 6D). These data point toward BAEC utilization of the ER store for supply of cytosolic Ca2+ during the EGCG challenge.

To critically evaluate the amount of Ca2+ supplied from the ER store to cytosol during the entire EGCG stimulus, we obtained cumulative Ca2+ responses. CPA-treated cells in comparison with control cells showed grossly reduced EGCG-induced cumulative Ca2+ responses (cumulative dF/F0 = 108 ± 18 and 654 ± 157%, respectively; t test, p < 0.01) (Fig. 6E). Therefore, BAEC respond to EGCG by an increase in Ca2+ store, showing an oscillatory pattern, and this Ca2+ excitability requires the supply of Ca2+ from the ER store. It should be noted, however, that the cumulative response is not completely blocked by CPA, which may indicate that other sources of Ca2+, such as the entry from the external space and mitochondria, might also contribute to EGCG-induced Ca2+ excitability; for discussion of Ca2+ sources, see Ref. 20. This intracellular Ca2+ contributed to EGCG-stimulated LC3-II formation because chelating intracellular Ca2+ by BAPTA-AM (10 μM, 30 min) or by the presence of extracellular EGTA (1 mM, 30 min) inhibited LC3-II formation (Fig. 6F).

**EGCG Facilitates Lipophagy**—It has been demonstrated that treatment with palmitate inhibits autophagic flux in pancreatic beta cells (38). So, we examined whether EGCG affects autophagic flux that was inhibited by palmitate. We treated BAEC with palmitate (200 μM, 4 h) in the presence or absence of lysosomal inhibitors (NH4Cl/Leu) (Fig. 7). As expected, the ratio of LC3-II/LC3-I was not further increased in the presence of the lysosomal inhibitor complex (NH4Cl/Leu) compared with the cells incubated with palmitate in the absence of lysosomal inhibitors (NH4Cl/Leu) (Fig. 7). In contrast, treatment of BAEC with EGCG and palmitate increased the ratio of LC3-II/LC3-I in the presence of NH4Cl/Leu compared with the cells treated without NH4Cl/Leu. The results suggest that palmitate inhibits lysosomal degradation of LC3-II, which is opposed by treatment with EGCG. Excess intake of lipid causes obesity and ectopic lipid accumulation, which is implicated as one of the causes for cardiometabolic syndrome (39–41). Fatty acid overload increases intracellular lipid droplets, and the presence of lipid droplets in non-adipose tissue plays a role in various pathophysiologies (42, 43). We examined whether EGCG-stimulated autophagic flux contributes to the reduction of intracel-
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DISCUSSION

This study demonstrates that EGCG stimulates autophagy through a CaMKKβ/AMPK-dependent mechanism and facilitates autophagic flux (Fig. 9). Furthermore, EGCG-stimulated lysosomal degradation leads to reduced accumulation of intracellular lipid droplets in vascular endothelial cells. These EGCG effects in vascular endothelium may contribute to protection from lipid-mediated endothelial dysfunction and cardiovascular complications.

EGCG Induces Autophagy through AMPK and CaMKKβ—In this study, we demonstrate that EGCG induces autophagosome and autophagolysosome formation (Fig. 2) through a CaMKKβ/AMPK signaling pathway leading to facilitation of autophagic flux (Figs. 3 and 5).

Previously, it has been shown that a high concentration (50–100 μM) of EGCG stimulates autophagy leading to cell death in cancer cells (44, 45). Another study reported that EGCG stimulates autophagy, which leads to inhibition of endotoxin-induced septic shock through EGCG-induced degradation of HMGB1, a late lethal inflammatory factor (46). However, a molecular mechanism for the EGCG-stimulated autophagy with regard to Ca^2+ /CaMKKβ and lipid droplet is unknown. In this study, we demonstrate a novel mechanism for EGCG-stimulated autophagy and its functional consequence in degradation of lipid droplets.

AMPK is a key mediator for the initial process of autophagy by stimulating the phosphorylation of ULK and formation of its protein complex with multiple autophagic proteins (47). Thus, activation of AMPK is crucial for initiation of autophagy. Because AMPK is an energy-sensing enzyme recognizing the AMP/ATP ratio, starvation conditions activate autophagy through an AMPK-dependent mechanism (48). Thus, EGCG may be mimicking starvation or caloric restriction conditions, which are consistent with the beneficial health effects of polyphenols, including EGCG and resveratrol (49–52). EGCG also has an anti-diabetic effect that is similar to metformin, an anti-diabetic drug that activates AMPK (53). This suggests that EGCG and metformin may have a common mechanism to ameliorate metabolic and cardiovascular disorders. Knockdown of ATG5 or CaMKKβ was significantly but not completely able to block the LC3-II formation (Figs. 1E and 5D). This may be due to the incomplete removal of ATG5 or CaMKKβ, because siRNAs were able to knock down only 40 and 48% of ATG5 and CaMKKβ, respectively. However, it is possible that the remaining LC3-II after knockdown of ATG5 or CaMKKβ could be due to the CaMKKβ- or ATG5-independent mechanism. Using primary cells from knock-out mice may help to understand the precise mechanism. In this study, our results suggest that EGCG stimulates LC3-II formation at least in part through an ATG5- and CaMKKβ-mediated mechanism.

We observed that EGCG activates AMPK, whereas EGCG did not stimulate the phosphorylation of mTOR (Fig. 5C). Activation of AMPK inhibits mTOR in starvation-induced autophagy (54, 55). However, we were not able to observe that mTOR is inhibited by EGCG, which may be a cell type-specific...
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or a stimulus-specific response. Interestingly, we observed that EGCG was not able to induce LC3-II formation in mouse embryonic fibroblasts (data not shown). In addition, other studies have shown that palmitate induces autophagy through an mTOR-independent mechanism (56). This unexpected result suggests that EGCG-stimulated accumulation of LC3-II is independent of the mTOR pathway.

**EGCG Induces Cytosolic Ca\(^{2+}\) Dynamics Implicated in Autophagy—**EGCG induces intracellular Ca\(^{2+}\) dynamics, and chelating cytosolic calcium by BAPTA-AM or reducing avail-
that the elevated cytosolic Ca$^{2+}$ treatment with CPA, a blocker of ER Ca$^{2+}$ (Fig. 6), EGCG stimulates Ca$^{2+}$ levels (52). However, the link between autophagy and the activation of ryanodine receptor to increase intracellular Ca$^{2+}$ is not known. Here, EGCG-induced autophagy is increased in the EGCG-treated cells when lysosomal degradation was blocked (Fig. 8). These suggest that treatment with palmitate seems to inhibit the fusion process of autophagosome and lysosome, and the fusion is facilitated by EGCG.

EGCG Facilitates Lipophagy—In this study, we demonstrate that EGCG facilitates autophagic flux, which contributes to oppose palmitate-induced accumulation of lipid droplets in endothelial cells (Figs. 7 and 8). Reduction of lipid droplets in endothelial cells through autophagic flux suggests a novel mechanism for EGCG-mediated beneficial health effects. Autophagy (or lipophagy) plays a role in lipid metabolism both in adipose tissue and in non-adipose tissue (42, 43, 62). Reduction of lipid accumulation in adipose tissue leads to weight loss and improvement of whole body metabolism. In contrast, reduced lipid accumulation in vascular endothelium may contribute to cardiovascular function, because triglyceride and cholesterol are the major components of lipid droplets that are associated with atherosclerosis and coronary heart disease. Chronic high fat diet and acute high cholesterol diet lead to impaired lysosomal degradation (63), and fatty acid inhibits autophagic flux due to the failure of lysosomal degradation in beta cells (38). Consistent with these reports, our data show that palmitate impairs autophagic flux in primary aortic endothelial cells, and EGCG enhances degradation of lipid droplets through facilitation of autophagic flux.

Despite the reduced number of lipid droplets in the EGCG-treated cells, a larger number of lipid droplets co-localize with LC3 and LAMP-1 in the presence of NH$_4$Cl/Leu (Fig. 8). The reason for the smaller number of lipid droplets and the fewer co-localizations of lipid droplets and LC3 or LAMP-1 in the presence of EGCG without NH$_4$Cl/Leu seems to be due to the rapid degradation of lipid droplets.

The accumulation of lipid droplets is much more prominent in the cells treated with palmitate alone than the cells treated with palmitate along with EGCG. One possibility is that triglyceride synthesis could be slower in EGCG-treated cells. However, we observed that reduction of the lipid droplet was almost identical whether the cells were co-treated or post-treated with EGCG with palmitate (Fig. 8, H and I). Moreover, co-localization of lipid droplets with autophagosomes was markedly increased in the EGCG-treated cells when lysosomal degradation was blocked (Fig. 8). These suggest that treatment with palmitate seems to inhibit the fusion process of autophagosome and lysosome, and the fusion is facilitated by EGCG.

Autophagic flux can be divided into three steps as follows: (i) formation of autophagosome; (ii) formation of autolysosome by fusion of lysosome and autophagosome; and (iii) lysosomal degradation. Formation of autophagosome was dramatically increased by EGCG as shown the samples with lysosomal inhibitors (Fig. 3, A and B, 2nd and 4th lanes), and lysosomal degradation was increased (Fig. 3, C–E). Nonetheless, our results suggest that EGCG stimulates lipophagy through facilitation of autophagosome formation, lysosomal fusion, and degradation. This does not exclude the possibility that other lipases, including hormone-sensitive lipase and endothelial lipase, may contribute to reduction of lipid accumulation. In fact, EGCG stimulates hormone-sensitive lipase in adipocytes and pancreatic
lipases in the serum that are associated with weight loss in adipose tissue (64, 65). In contrast, our data present for the first time that EGCG reduces endothelial ectopic lipid accumulation. We previously reported that EGCG intake protects from insulin resistance, hypertension, and ischemia-reperfusion injury in the heart in spontaneously hypertensive rats (12). These beneficial health effects of EGCG in cardiovascular tissues may be associated with the EGCG-induced facilitation of autophagy. Further studies are required to understand the more detailed regulatory mechanisms for EGCG-stimulated autophagic flux.

In summary, EGCG induces autophagy through a $Ca^{2+}$/CaMKKβ/AMPK-mediated mechanism, which contributes to reduction in the palmitate-induced accumulation of lipid droplets in endothelial cells. These findings suggest the following: 1) heightened intracellular calcium dynamics activating CaMKKβ/AMPK may play an important role in the beneficial health effect of green tea; 2) EGCG stimulates autophagic flux, a key step for autophagic degradation, which may help reduce the accumulation of lipid; 3) supplementation of green tea may have the beneficial effect in endothelial function through facilitation of lipolysis. These effects of green tea polyphenol may help prevent metabolic and cardiovasculardisorders.

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