Epigallocatechin gallate increases the formation of cytosolic lipid droplets and decreases the secretion of apoB-100 VLDL

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Abstract **Epigallocatechin gallate (EGCG) increases the formation of cytosolic lipid droplets by a mechanism that is independent of the rate of triglyceride biosynthesis and involves an enhanced fusion between lipid droplets, a process that is crucial for their growth in size. EGCG treatment reduced the secretion of both triglycerides and apolipoprotein B-100 (apoB-100) VLDLs but not of transferrin, albumin, or total proteins, indicating that EGCG diverts triglycerides from VLDL assembly to storage in the cytosol. This is further supported by the observed increase in both intracellular degradation of apoB-100 and ubiquitination of the protein (indicative of increased proteasomal degradation) in EGCG-treated cells. EGCG did not interfere with the microsomal triglyceride transfer protein, and the effect of EGCG on the secretion of VLDLs was found to be independent of the LDL receptor. Thus, our results indicate that EGCG promotes the accumulation of triglycerides in cytosolic lipid droplets, thereby diverting lipids from the assembly of VLDL to storage in the cytosol. Our results also indicate that the accumulation of lipids in the cytosol is not always associated with increased secretion of VLDL.—** Li, L., P. Stillemark-Billton, C. Beck, P. Boström, L. Andersson, M. Rutberg, J. Ericsson, B. Magnusson, D. Marchesan, A. Ljungberg, J. Borén, and S-O. Olofsson. **Epigallocatechin gallate increases the formation of cytosolic lipid droplets and decreases the secretion of apoB-100 VLDL.** ***J. Lipid Res.*** **2006.** 47: **67–77.**

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Accumulation of triglycerides, particularly in the liver and skeletal muscle, is associated with metabolic disorders such as insulin resistance and type 2 diabetes (1, 2), diseases that are strong risk factors for cardiovascular diseases. Accumulation of triglycerides in the liver is also the landmark of nonalcoholic fatty liver disease, which results in inflammation and liver damage (3). Moreover, the stores of triglyceride in the liver fuel the assembly and secretion of VLDLs (3–6). Thus, it is obvious that elucidation of the mechanism of the storage of lipid in the cytosol is important for our understanding of the pathogenesis of major metabolic diseases.

Triglycerides are stored in the cytosol in the form of lipid droplets (also referred to as lipid bodies) (for reviews, see 7, 8). These droplets are formed from microsomes (9; see also 7, 8 for reviews) as a primordial droplet with a diameter of ~0.1 μm (9), a process in which phospholipase D (PLD) and the generation of phosphatidic acid (PA) have important roles (9; L. Andersson, P. Boström, M. Rutberg, J. Ericsson, D. Marchesan, B. Magnusson, M. Ruiz, L. Asp, M. A. Frohman, J. Borén, and S-O. Olofsson, unpublished data). The primordial particles increase in size after their formation by a mechanism that is independent of the rate of triglyceride biosynthesis and that involves a fusion between individual droplets. Intact microtubules are essential for this process, and the cytosolic droplets contain the motor protein dynein (10).

It has been demonstrated that the fatty acids used for the biosynthesis of VLDL triglycerides are derived from triglycerides stored in the liver (3). The triglycerides are hydrolyzed and reesterified into new triglycerides before being assembled into VLDL (4–6). VLDLs are assembled in a complex series of events (for reviews, see 11–16), starting with the formation of a primordial particle (pre-VLDL) during the translation and translocation of apolipoprotein B-100 (apoB-100) to the lumen of the endoplasmic reticulum. This step is catalyzed by the microsomal triglyceride transfer protein (MTP). The pre-VLDL par-

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Abbreviations: apoB-100, apolipoprotein B-100; EGCG, epigallocatechin gallate; GFP, green fluorescent protein; HAT, histidine affinity tag; LDLR, low density lipoprotein receptor; MTP, microsomal triglyceride transfer protein; PA, phosphatidic acid; PLD, phospholipase D.

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ticle must be further lipidated to a bona fide VLDL to be secreted (17). ApoB-100 that has not acquired enough lipids during the translation is retracted to the cytosol, ubiquitinated, and sorted to proteasomal degradation (18, 19). Because the secretion of the apoB-100-containing pre-VLDL is very low (17), it is likely that this particle is sorted to posttranslational degradation (18) unless it is not converted to VLDL (for reviews, see 11, 13, 15, 16).

To achieve a deeper understanding of the relationship between cytosolic triglycerides and the assembly of VLDL, one must investigate whether all triglycerides in the cytosol are available for VLDL assembly or whether there exist situations in which triglycerides are sequestered from VLDL assembly to be stored in the cytosol. To address this question and to continue to delineate the process involved in the formation of lipid droplets, we require tools to manipulate the triglyceride storage process experimentally. In our search for such tools, we came across epigallocatechin gallate (EGCG), a monomeric phenol of the catechin group that is found in green tea (20). It has been shown to activate PLD (21) and thus has the potential to stimulate the assembly of cytosolic lipid droplets (9; L. Andersson, P. Boström, M. Rutberg, J. Ericsson, D. Marchesan, B. Magnusson, M. Ruiz, L. Asp, M. A. Frohman, J. Borén, and S-O. Olofsson, unpublished data). Furthermore, EGCG has been shown to influence the secretion of apoB-100 in HepG2 cells (22).

Here, we report that EGCG strongly promotes the assembly of cytosolic lipid droplets by increasing the rate of fusion between these droplets. In addition, EGCG increases the ubiquitination and early (proteasomal) degradation of apoB-100 and prevents the assembly and secretion of VLDLs, especially VLDL1. Thus, our results indicate that lipid can be sequestered from the VLDL assembly process to storage in the cytosol; that is, not all cytosolic triglycerides are available for VLDL assembly.

MATERIALS AND METHODS

Eagle's minimum essential medium, nonessential amino acids, glutamine, penicillin, and streptomycin were obtained from ICN Biomedicals. Fetal calf serum was from Biochrom KG. EGCG, heparin, methionine, fatty acid-free BSA, sodium pyruvate, disodium carbonate, sodium hydrogen carbonate, phenylmethylsulfonyl fluoride, pepstatin A, and leupeptin were from Sigma. Rabbit immunoglobulin was from Dako, rabbit anti-rat transferrin IgG was from Organon Teknika, and rabbit anti-mouse albumin IgG was from Abcam. Trasylol (aprotinin) was from Bayer. Antibodies to ubiquitin were from Stressgene. Immunoprecipitin and Eagle's minimum essential medium without methionine were from Life Technologies. *N*-Acetyl-leu-leu-norleucinal was from Boehringer Mannheim. Amplify, [^{35}S]methionine/cysteine mix, [^{3}H]palmitic acid, and the Rainbow protein molecular weight marker system were from Amersham Pharmacia Biotech. Ready-Safe was from Beckman. All chemicals for SDS-PAGE were from Bio-Rad.

Cell culture

McA-RH7777 cells were cultured in the presence of oleic acid (unless stated otherwise) as described previously (23). The cultures were split twice weekly and fed daily. Hepatocytes from mice were prepared as described (17). Briefly, the livers were perfused through the portal vein, first with calcium- and magnesium-free Hank's balanced salt solution, pH 7.4, supplemented with 0.6 mM EGTA, 20 mM HEPES, and 10 mM sodium hydrogen carbonate (7–8 min, 40–50 ml/min), and then with Williams' E medium with Glutamax supplemented with penicillin (50,000 IU/l), streptomycin (50 mg/l), 0.28 mM sodium ascorbate, 0.1 μM sodium selenite, and 400 mg/l collagenase type IV (8–10 min, 40 ml/min). The perfusion media were kept at 37°C and infused continuously with 95% oxygen and 5% carbon dioxide. After filtration through a 250 μm pore size nylon mesh filter and then a 100 μm cell strainer, the cells were washed three times by centrifugation at 50*g* for 1 min each at 4°C in Williams' E medium with Glutamax containing glucose (3 g/l), insulin (Actrapid; 16 nM = ~91 μg/l = ~2,600 mU/l), and the antibiotics, sodium ascorbate, and selenite described above. The cells were seeded (70,000 cells/cm^2) in 10 ml of medium in Primaria cell culture dishes (58 cm^2). After 4 h, the medium was replaced with fresh medium, and the cells were cultured for 13 h before use. In preliminary experiments with hepatocytes from human apoB-100 transgenic mice, apoB-100 VLDL production started to decline after 3 days in culture and had almost ceased after 4 days.

NIH 3T3 cells were cultured as recommended by the American Type Culture Collection.

EGCG treatment

McA-RH7777 and NIH 3T3 cells were treated with EGCG (20 or 50 μM) for 2 h before and during labeling, or as indicated. In some experiments, primary hepatocytes were isolated from mice that had received intraperitoneal injections of EGCG (1 mg/day) for 7 days.

Quantification of lipid droplet formation

To investigate the role of EGCG in lipid droplet formation, we used NIH 3T3 and McA-RH7777 cells. Intact cells were fixed in 3.7% formaldehyde for 10 min, washed with 60% isopropanol for 30 s, and stained with Oil Red O in 60% isopropanol for 20 min. The cells were again washed with 60% isopropanol for 30 s, stained with hematoxylin for 20 min, washed with cold water, and viewed with a Zeiss epifluorescence microscope. Ten to 20 images were captured and digitized (TIFF format, 8 pixels/μm) and analyzed with BioPix software (www.biopix.se), which categorizes the pixels as red or nonred; adjacent red pixels are identified as a lipid droplet, as described (24). The program can determine the number of droplets, the total lipid droplet area per cell, and the number of droplets in different size categories (L. Andersson, P. Boström, M. Rutberg, J. Ericsson, D. Marchesan, B. Magnusson, M. Ruiz, L. Asp, M. A. Frohman, J. Borén, and S-O. Olofsson, unpublished data).

Quantification of the rate of fusion between lipid droplets

The cell-free system was that recently described (10), with the exception that adipocyte differentiation related protein (ADRP) was fused with a histidine affinity tag (HAT) instead of green fluorescent protein (GFP) and Talon Dynabeads (Dynal) were used to precipitate lipid droplets containing ADRP-HAT. The protocol was that recommended by the manufacturer. To control for unspecific binding to the precipitation system, we used GFP fused to the HAT. Only minor amounts (6 ± 4%; mean ± SD, n = 3) of radiolabeled triglyceride present in the system could be precipitated after the incubation of the system, when the GFP-HAT construct was used to transfect the cells instead of ADRP-HAT.

To construct ADRP-HAT, cDNA of mouse ADRP mRNA was amplified from NIH 3T3 cells by PCR using primers 5′-GTCGACATGGGAGCAGCAGTAGTGGA-3′ and 5′-GTCGACTCTAGAATTACTGAGCTTTGACCTC-3′. The PCR product was cloned in pHAT10 (BD Biosciences) via PCR*T*2.1-TOPO (Invitrogen). A kozak site was first introduced into pHAT10 by means of the following primers: 5′-AAATTTAAGCTTCCACCATGGGAAAGGATCATCTCATCCACAA-3′ and 5′-GAATTCTCAGCTAATTACGA-3′. ADRP-HAT was finally excised and subcloned in pcDNA4/TO (Invitrogen).

GFP was amplified from pcDNA-DEST53 Gateway*T* Vector (Invitrogen) using the primers 5′-TTTGGTACCATGGCCAGCAAAGGAGAAGA-3′ and 5′-TTTGAATTCTAGATTATTTGTAGAGCTCATCCA-3′ and cloned in pHAT10 with a kozak site. HAT-GFP was amplified using the primers 5′-AAATTTAAGCTTCCACCATGGGAAAGGATCATCTCATCCACAA-3′ and 5′-TTTGAATTCTAGATTATTTGTAGAGCTCATCCA-3′ and subcloned in pcDNA4/TO via PCR*T*2.1-TOPO.

The rate of fusion between lipid droplets was also estimated as the increase in size of formed droplets when the triglyceride biosynthesis was blocked with triacsin C, using a recently described protocol (10). We also estimated the frequency of fusions during time-lapse studies as described previously (10). In these experiments, the cells were either microinjected with the ADRP-GFP construct or stained with Bodipy or Nile Red (10).

Metabolic labeling and lipoprotein separation

The cells were pulse-labeled and chased as described previously (25). Cells and the microsomal fraction were isolated as described (26). The luminal content of the vesicles was separated from the vesicle membranes by the sodium carbonate deoxycholate method (25). Sucrose gradient ultracentrifugation was carried out as described (17, 23). ApoB was isolated by immunoprecipitation and SDS-PAGE (25).

To estimate triglyceride biosynthesis and secretion, the cells were incubated with [^{3}H]palmitic acid and the radioactivity was determined as described (27). The rate of β-oxidation was measured as the production of radiolabeled ketone bodies from [^{3}H]palmitic acid (28).

The incorporation of radioactivity into PA and lyso-PA was determined after 24 h of incubation with [^{3}H]palmitate (1 μCi/ml culture medium). The lipids were extracted from the cells using chloroform-methanol as described (27), and the PA and lyso-PA was isolated by two-dimensional thin-layer chromatography as described previously (29).

Ubiquitinated apoB-100 was isolated as described (30). The cells were radiolabeled for 2 h and immunoprecipitated with anti-ubiquitin antibodies. The isolated precipitate was reprecipitated with antibodies to apoB-100, and the final precipitate was analyzed by SDS-PAGE and autoradiography.

Lactacystin treatment was carried out as described (31).

Other methods

PLD activity was quantified as described (21). Briefly, the cells were labeled with [^{3}H]myristic acid for 12 h and incubated first with EGCG (as indicated) and then with 0.3% 1-butanol for 50 min. The cells were harvested and extracted with organic solvents and isolated by TLC.

MTP activity was estimated using the fluorescent MTP activity kit assay (Roar Biomedical, New York, NY) (18, 32). Briefly, cells were sonicated in the buffer specified by the manufacturer and incubated at 37°C with donor and acceptor particles. The effect of EGCG was investigated by using it to treat cell homogenate from McA-RH7777 cells. EGCG was also added to the assay system using homogenate from both treated and untreated cells. Moreover, EGCG was tested in assays with a partially purified MTP (provided by the manufacturer).

SDS-PAGE and immunoblot analysis were carried out as described previously (27).

Groups were compared with the Mann-Whitney rank sum test or *t*-test, whereas one-way ANOVA was used to compare multiple groups. All experiments reported were repeated once, unless stated otherwise.

RESULTS

EGCG increases the level of cytosolic lipid droplet assembly

In NIH 3T3 cells (a cell line that does not secrete VLDL), EGCG increased the total area of Oil Red O-stained droplets/cell by 3.2 ± 1.2-fold (mean ± SD of four experiments). In each of these experiments, there was a highly significant difference between the control and the EGCG-treated group (*P* values between 0.001 and 0.002) (one example is shown in **Fig. 1A, B**).

In similar experiments with McA-RH7777 cells (a cell line that does secrete VLDL), EGCG increased the total area of Oil Red O-stained droplets/cell by 4.5 ± 2.5-fold (mean ± SD of four experiments). In each of these experiments, there was a highly significant difference between the control and the EGCG-treated cells (*P* values all <0.002). One example is given in Fig. 1C, D. EGCG activity was maximal after 2 h of treatment, and droplet formation did not increase further after 4 and 8 h (data not shown).

EGCG also promoted triglyceride retention in McA-RH7777 cells, as shown after a 5 h pulse with [^{3}H]palmitic acid followed by a 3 h chase (**Fig. 2A**). The secretion of triglycerides had reached a plateau level after the 3 h chase. EGCG also reduced the triglyceride secretion (Fig. 2B).

EGCG did not affect the rate of triglyceride biosynthesis in McA-RH7777 cells, as assessed by the incorporation of [^{3}H]palmitic acid into triglycerides over 30 min (Fig. 2C) or the β-oxidation of palmitic acid in McA-RH7777 cells or mouse hepatocytes (data not shown).

Next, we investigated whether EGCG resulted in a change in the degradation of triglycerides. The cells were labeled with [^{3}H]palmitic acid for 12 h and than chased in the presence of triacsin C to prevent the biosynthesis of triglycerides and the reesterification of released fatty acids into new triglycerides. The decrease in the labeled triglyceride pool was followed during this chase. The results (illustrated by the observations in NIH 3T3 cells in Fig. 2D) demonstrated that neither in NIH 3T3 cells nor in McA-RH7777 cells did EGCG prevent the degradation of cellular triglycerides.

In these two cell systems, we could not detect any effect of EGCG on PLD activity. This is supported by the observation that treatment with EGCG failed to increase the amount of radioactive PA recovered from the cell after a 2 h incubation with [^{3}H]palmitic acid (data not shown). We also investigated the accumulation of lyso-PA but could not detect any difference in the EGCG-treated cells (data not shown).

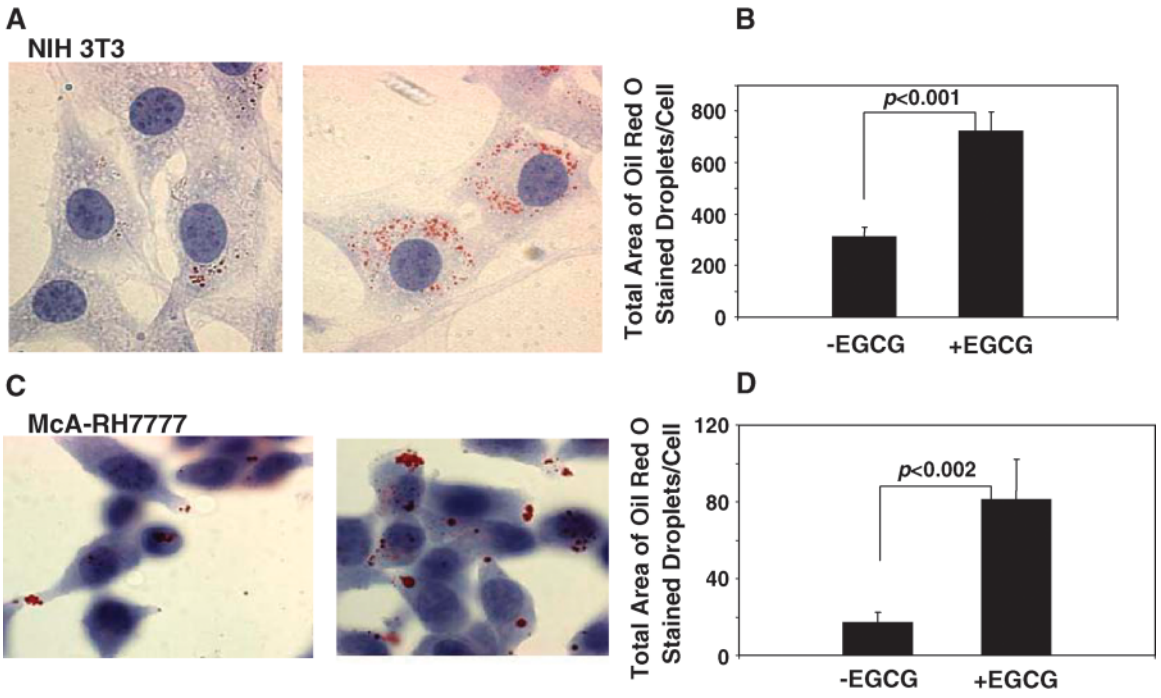

Fig. 1. Epigallocatechin gallate (EGCG) increases the amount of cytosolic lipid droplets. NIH 3T3 cells (A, B) and McA-RH7777 cells (C, D) were incubated in the absence or presence of EGCG for 2 h and stained with Oil Red O (A, C), and the total area of Oil Red O-stained lipid droplets was calculated with BioPix software (B, D). Values are means ± SEM of all cells in 10 randomly selected images of the cell culture. (Mann-Whitney rank-sum test).

We recently demonstrated that cytosolic lipid droplets increase in size by a fusion process (10). To investigate whether EGCG could influence this fusion process, we first investigated the effect of the substance in our recently described cell-free system (10). In this system, cell homogenates from two cell cultures are mixed; the first was incubated with [^{3}H]palmitic acid to obtain cytosolic lipid droplets with radiolabeled triglycerides, and the second cell culture was transfected with a HAT-tagged ADRP, which was used for precipitation of droplets. The accumulation of radiolabeled triglycerides (present in lipid droplets in the first cell culture) in droplets with tagged ADRP (from the second cell culture) is taken as a measure of the rate of fusion between droplets (10). The results demonstrate that EGCG strongly promotes the fusion between lipid droplets in this cell-free system (**Fig. 3A).**

In the second type of experiment, we used intact cells as described previously (10). In these experiments, the cells were incubated with oleic acid for 2 h to induce the formation of lipid droplets and then chased for 2 h in the presence of triacsin C or triacsin C together with EGCG. Triacsin C completely inhibited triglyceride biosynthesis (10); thus, an increase in the size of the droplets is attributable to a growth of the droplets that is independent of any inflow of triglycerides into the system. As shown previously (10), the major proportion of the lipid droplets present in the cell after the incubation with oleic acid had a relatively small diameter: in these experiments, 0.5–2 μm (Fig. 3B, fraction I). Also in agreement with previous results (10), there was a decrease in the proportion of droplets present in this size range when the cells were chased in the presence of triacsin C (Fig. 3B, fraction I); instead, there was a shift in size toward droplets with larger diameter (Fig. 3B, fraction II). This decrease in fraction I and subsequent increase in fraction II was enhanced when the chase was carried out in the presence of triacsin C and EGCG (Fig. 3B). To study the effect of EGCG in more detail, we carried out four separate experiments in which the cells were chased in the presence of triacsin C, or in the presence of triacsin C together with EGCG, and the proportions of droplets recovered in fractions I and II (Fig. 3B) were monitored. The results demonstrate that the chase in the presence of both triacsin C and EGCG gave rise to a significant reduction in the proportion of droplets in fraction I (droplets with a diameter between 0.5 and 2 μm) and a subsequent increase in the proportion of droplets in fraction II (the larger droplets) compared with cells chased in the presence of triacsin C alone (Fig. 3C). There was no difference in the total area of Oil Red O-stained lipid droplets under the two conditions (data not shown). These results support the conclusion that EGCG promotes the triglyceride-independent increase in the size of the droplets (i.e., the fusion between these droplets) (10).

Finally we followed the fusion process by counting the observed fusion during time-lapse experiments. The results showed a frequency of fusion events similar to that described previously (10). There was a significant increase in the frequency of fusion events (2.7 ± 1.4-fold; mean ± SD, n = 4) in cells treated with EGCG (Fig. 3D).

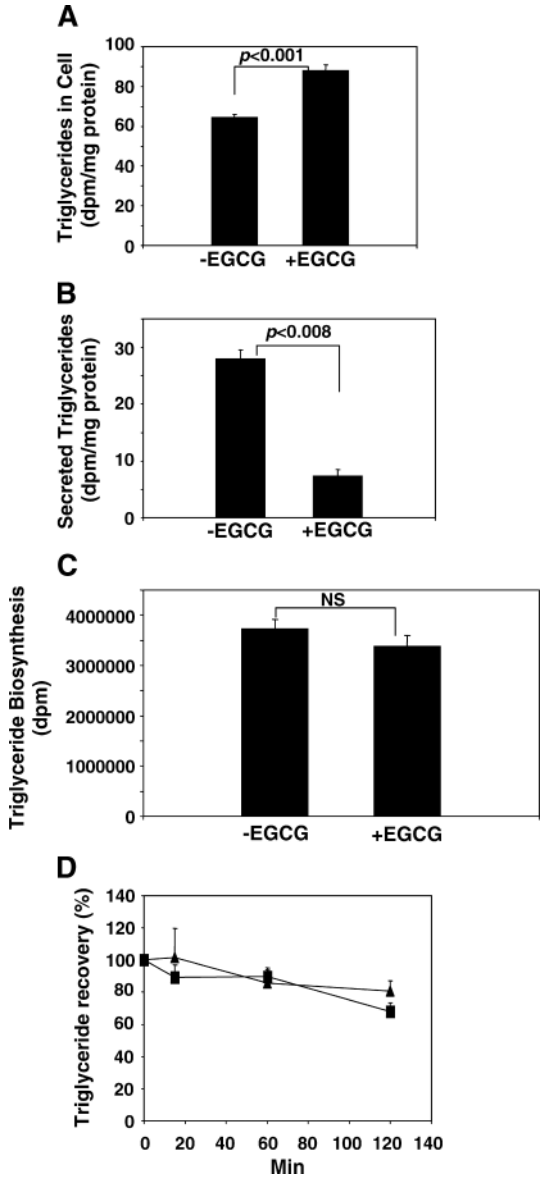

Fig. 2. EGCG increases cytosolic triglyceride content and reduces triglyceride secretion but does not affect triglyceride biosynthesis or turnover. A: McA-RH7777 cells were labeled with [^{3}H]palmitic acid for 5 h and chased for 3 h. The cells were recovered, homogenized, and centrifuged at low speed, and the radioactivity of triglycerides in microsomes and cytosol was determined. Values are means ± SD (n = 3 culture dishes; Mann-Whitney rank-sum test). B: McA-RH7777 cells were labeled and chased as in A, the medium was collected, and the radioactivity in triglycerides was determined. Values are means ± SD (n = 5 culture dishes; Mann-Whitney rank-sum test). C: Effect of EGCG on triglyceride biosynthesis. McA-RH7777 cells were incubated with [^{3}H]palmitic acid for 30 min, and the intracellular accumulation of radioactive triglycerides was determined. Values are means ± SD of three culture dishes. D: Effect of EGCG on the turnover of triglycerides in NIH 3T3 cells. Cells were labeled with [^{3}H]palmitic acid for 12 h and then chased in the presence of triacsin C [which completely blocks the biosynthesis of triglycerides in these cells (10)] and in the presence (triangles) or absence (squares) of EGCG. After each chase period, the cells were lysed, the triglycerides were recovered by thin-layer chromatography, and the radioactivity was determined. Results are given as percentage of the radioactivity present after the labeling period (means ± SD; n = 3 culture dishes).

Together, our results indicate that EGCG promotes the formation of lipid droplets by enhancing the rate at which they grow by fusion. Thus, EGCG is a potentially important tool in the elucidation of the mechanism behind the assembly of lipid droplets.

EGCG reduces the secretion of apoB-100 VLDL

In the next experiment, we investigated whether the EGCG-driven assembly of cytosolic lipid droplets increases or decreases the secretion of apoB-100 and VLDL in McA-RH7777 cells and primary mouse hepatocytes. In McA-RH7777 cells, treatment with EGCG for 2 h reduced the secretion of apoB-100-containing VLDL1, the total amount of apoB-100, and the amount of apoB-100 in less buoyant particles (LDL to VLDL2 density region) (**Fig. 4A, B**) during a 30-min pulse and a 3 h chase; secretion of transferrin (Fig. 4B) and total proteins (data not shown) was not significantly affected. We also investigated the secretion of apoB-100 VLDL during a 2 h labeling, when steady-state labeling of the total apoB-100 pool in the cell had been reached. Under these conditions, total apoB-100 secretion decreased by 55 ± 5% (mean ± SD) after treatment with EGCG.

In primary hepatocytes from two human apoB-100 transgenic mice treated with EGCG for 7 days, exposure to EGCG during the plating and the pulse and chase markedly reduced the secretion of apoB-100 VLDL (**Fig. 5A**), with no difference between cells from the two mice. This reduction required EGCG in the culture medium. In these experiments, the cells were labeled for 2 h to achieve steady-state labeling of apoB-100 and then chased for 3 h. Under these conditions, the whole secretable pool of apoB-100 was recovered in the medium. Albumin secretion was not significantly affected (Fig. 5B).

Together, these results indicate that the EGCG-induced formation of cytosolic lipid droplets reduces the assembly of VLDLs, in turn indicating that triglycerides are diverted from the mechanism of VLDL assembly.

EGCG increases the intracellular degradation of apoB-100

If EGCG diverts triglycerides from the assembly pathway to cytosolic lipid droplets, the intracellular degradation of apoB-100 should increase (reviewed in 11, 13). Because proteasomal degradation is one of the ways through which apoB-100 is removed (18), we first investigated the effects of EGCG on the ubiquitination of apoB-100 in McA-RH7777 cells. Ubiquitination was increased (**Fig. 6A**). Next, we investigated whether apoB-100 was lost in the cell. Pulse-chase studies demonstrated that EGCG promoted a loss of apoB-100 already after the labeling period (15 min) (Fig. 6B), which is consistent with an early degradation of the protein (and the increased ubiquitination). Because EGCG did not inhibit the secretion of transferrin, albumin, or total proteins (see above), the loss of apoB-100 does not reflect a general inhibition of protein biosynthesis.

These results, indicating increased proteasomal degradation of apoB-100 in cells treated with EGCG, are con-

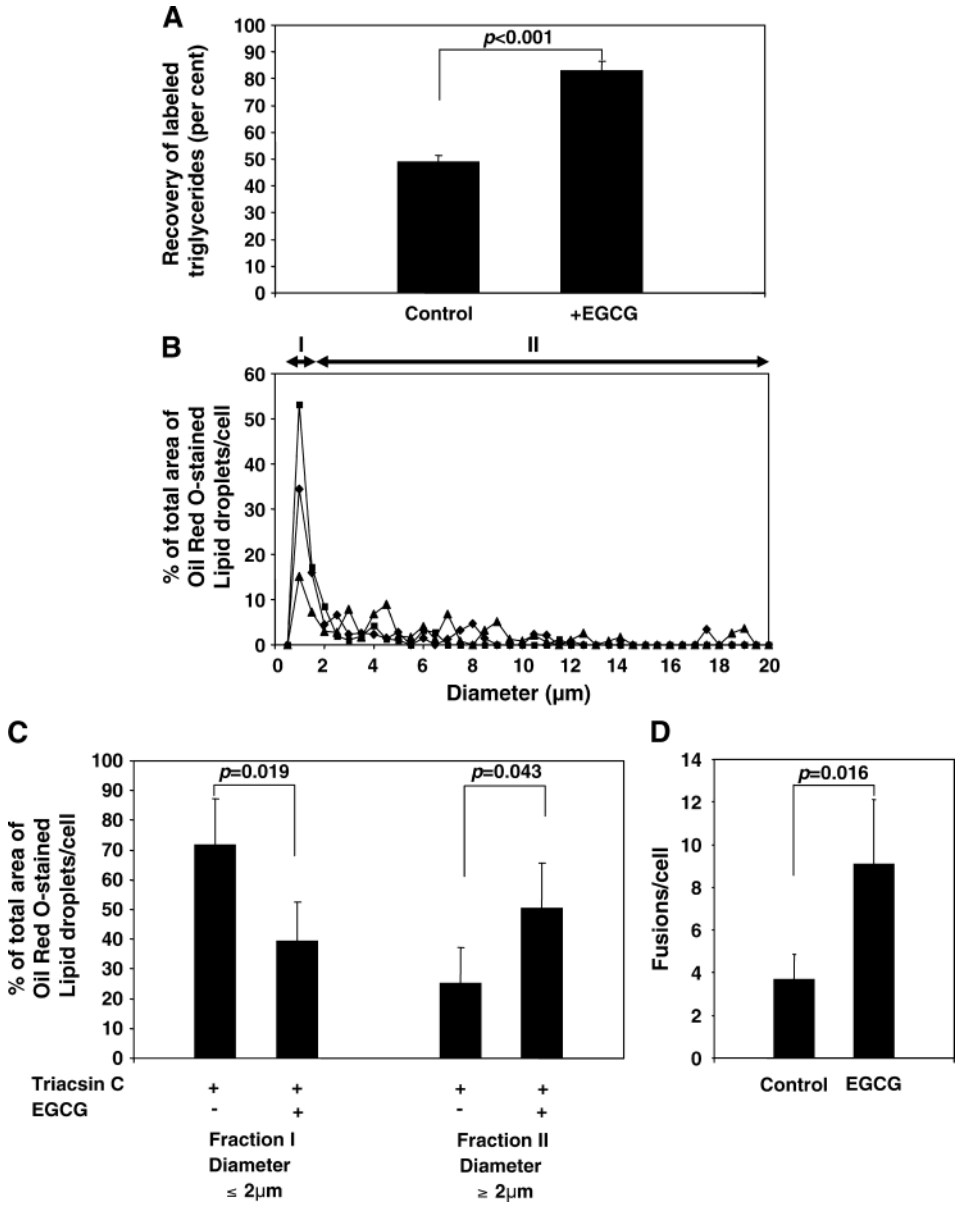

Fig. 3. EGCG increases the rate of fusion of lipid droplets. A: EGCG increases the rate of fusion between lipid droplets in a cell-free system. One cell culture of NIH 3T3 cells was transfected with ADRP-histidine affinity tag (HAT), and another culture was labeled for 2 h with [^{3}H]palmitic acid. The postnuclear supernatants from these cell cultures were mixed and incubated for 1 h in the presence (+EGCG) or absence (Control) of EGCG. After the incubation, the ADRP-HAT droplets were precipitated using Talon Dynabeads and the radiolabeled triglycerides were measured (see 10 for further details). Results are means ± SD (n = 6; *t*-test). B, C: EGCG enhances the disappearance of smaller droplets and the appearance of larger droplets in intact NIH 3T3 cells. B: NIH 3T3 cells were incubated with oleic acid for 2 h (squares) and chased for 2 h in the presence of triascine C alone (diamonds) or triascine C together with EGCG (triangles). The cells were stained with Oil Red O, and BioPix software was used to calculate the size distribution of the lipid droplets in the cell (percentage of the total area of lipid droplets per cell that were found within the different size ranges). C: Cells were incubated with oleic acid and chased in the presence of triacsine C alone or triacsine C together with EGCG for 2 h, as indicated. The distribution of the total area of Oil Red O-stained lipid droplets between fraction I (the size of the major amount of droplets present after the oleic acid pulse; see B) and fraction II (the larger droplets; see B) was determined. Results are means ± SD (n = 4; *t*-test). D: EGCG increases the rate of fusion in intact cells. Cells were either microinjected with ADRP-green fluorescent protein (into the nucleus) or stained with Nile Red and then imaged by confocal microscopy at intervals of 30 s for 5 min. The microinjection was carried out 2 h before the recordings started, whereas the Nile Red staining was done immediately before the start of the time-lapse experiments. The cells were incubated with oleic acid for 2 h before the recordings started. Three-dimensional reconstructions of the recordings were carried out as described (10), and the fusion events were identified (10). The results represent fusion events/cell (means ± SD) identified during 5 min in four different experiments (*t*-test).

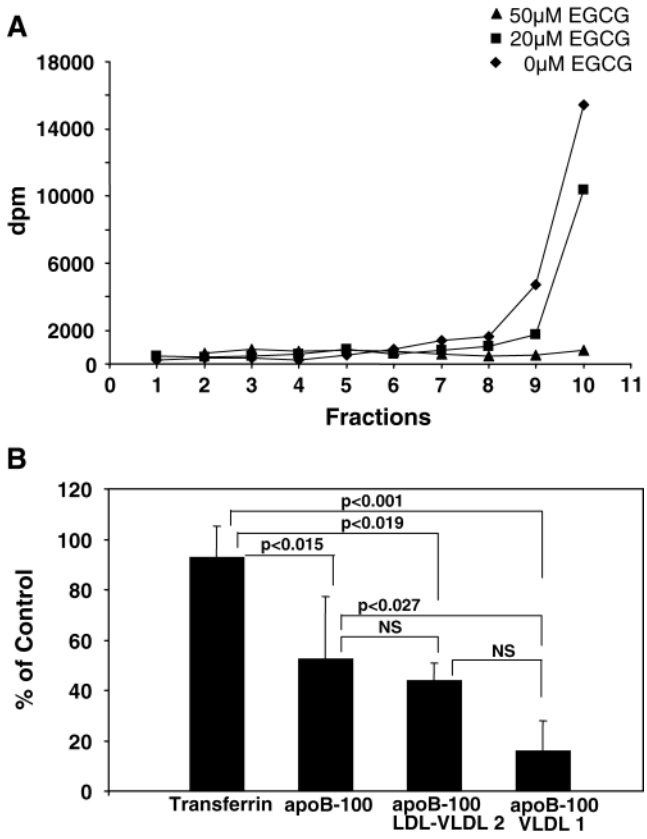

Fig. 4. EGCG reduces the secretion of apolipoprotein B-100 (apoB-100)-containing lipoproteins. McA-RH7777 cells were incubated with EGCG at 0, 20, and 50 μM (A) or 0 and 50 μM (B) for 2 h, pulse-labeled with [^{35}S]methionine/cysteine for 30 min, and chased for 180 min in the presence or absence of EGCG. A: The medium was collected and subjected to gradient ultracentrifugation. ApoB-100 was recovered from each fraction by immunoprecipitation and SDS-PAGE, and the radioactivity was determined. B: Transferrin and apoB-100 were recovered by immunoprecipitation and SDS-PAGE, and the radioactivity was determined. HDL, LDL-VLDL2, and VLDL1 were isolated by sucrose gradient ultracentrifugation, apoB-100 was recovered by immunoprecipitation and SDS-PAGE, and the radioactivity was determined. Values are means ± SD of three to eight experiments, each consisting of one culture dish and its control. *P* values were determined by ANOVA.

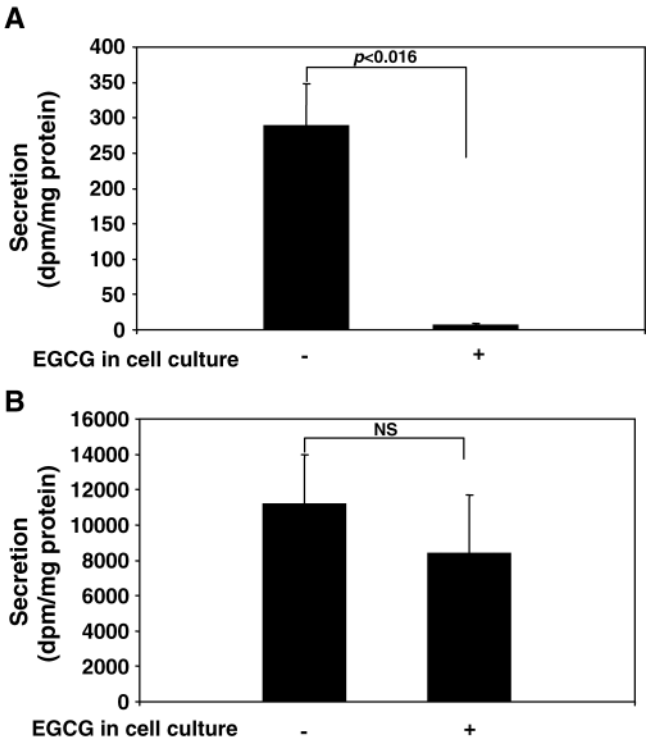

Fig. 5. Secretion of apoB-100 (A) and albumin (B) by hepatocytes isolated from two human apoB-100 transgenic mice that had received daily intraperitoneal injections of EGCG for 1 week. The cells were cultured overnight in the presence or absence of EGCG (50 μM), labeled with [^{35}S]methionine/cysteine for 120 min, and chased for 180 min in the presence or absence of EGCG. The medium was recovered, and albumin was isolated by immunoprecipitation and SDS-PAGE; VLDL was recovered by gradient ultracentrifugation, and apoB-100 was isolated by immunoprecipitation and SDS-PAGE. The radioactivity in apoB-100 and albumin was determined. Values are means ± SD; n = 5 culture dishes (apoB-100) and 4 culture dishes (albumin) (Mann-Whitney rank-sum test).

sistent with an EGCG-driven diversion of triglycerides from the assembly of VLDL to storage in cytosolic lipid droplets.

A diversion of lipids from the assembly pathway and a consecutive increased proteasomal degradation of apoB-100 could be caused by inhibition of the MTP. Therefore, we investigated whether EGCG might inhibit MTP. Our results indicated that this was not the case; if anything, there was a slight increase in the activity (data not shown).

To further test the role of proteasomes in the EGCG-induced degradation of apoB-100; the cells were treated with the proteasome inhibitor lactacystin, alone or together with EGCG, as well as with EGCG alone. Lactacystin treatment increased the secretion of apoB-100 (Fig. 6C), whereas EGCG treatment resulted in a decrease in this secretion. In cells treated with both EGCG and lactacystin, secretion of apoB-100 was greater than in those treated with EGCG alone; however, it did not reach the levels seen with only lactacystin. Thus, inhibition of the proteasome only partially blocked the effects of EGCG. This indicates that the shift of triglycerides from the assembly of VLDL to storage in the cytosol also influences the nonproteasomal degradation of the apoB-100.

LDL receptor does not mediate the effect of EGCG on apoB-100 secretion

Green tea induces expression of the low density lipoprotein receptor (LDLR) (33), an important determinant of the apoB secretion rate (34–37). To determine whether the effect of EGCG on the secretion of apoB-100 VLDL might be mediated by the LDLR, we first investigated the effect of EGCG in McA-RH7777 cells in the presence or absence of heparin (10 mg/ml during the chase). Because heparin displaces apoB-100-containing lipoproteins from the LDLR, we could ascertain whether reuptake of secreted apoB-100 VLDL might explain the effect of EGCG. Heparin did not alter the effect of EGCG on apoB-100 secretion (**Fig. 7A**).

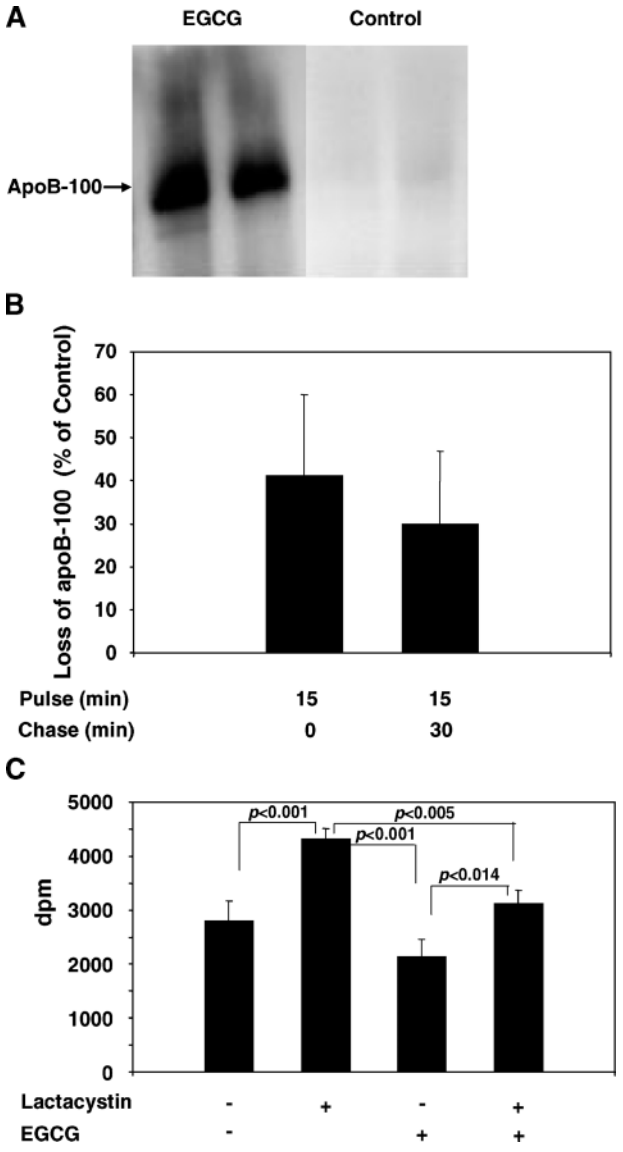

Fig. 6. EGCG increases the ubiquitination and intracellular degradation of apoB-100, which can be partly overcome by the proteasome inhibitor lactacystin. A: McA-RH7777 cells were incubated with 50 μM EGCG for 2 h (or left untreated) and labeled with [^{35}S]methionine/cysteine for 2 h in the presence or absence of EGCG. The cells were lysed, and ubiquitinated proteins were isolated by immunoprecipitation. ApoB-100 was recovered by immunoprecipitation and analyzed by SDS-PAGE. B: McA-RH7777 cells were treated with EGCG (50 μM) for 2 h (or left untreated), pulse-labeled with [^{35}S]methionine/cysteine for 15 min, and chased for 0 and 30 min. After each chase, the cells were lysed, apoB-100 was recovered by immunoprecipitation and SDS-PAGE, and the radioactivity was determined. Values are given as the percentage of radioactive apoB-100 that had disappeared in cells treated with EGCG compared with the control cells, which were set to 100%. Values are means ± SD (n = 5 treated dishes and 5 control dishes). There was no significant difference between the 0 and 30 min chase results. C: McA-RH7777 cells were treated with EGCG for 1 h and then with EGCG or lactacystin or both for 1 h, pulsed with [^{35}S]methionine/cysteine for 15 min, and chased for 3 h in the presence or absence of EGCG and/or lactacystin. The medium was collected, apoB-100 was recovered by immunoprecipitation and SDS-PAGE, and the radioactivity was determined. Values are means ± SD (n = 3 culture dishes). Significance was determined by ANOVA.

Next, we used human apoB-100 transgenic mice crossed with LDLR-deficient mice to assess the effect of EGCG on the secretion of apoB-100 in the presence and absence of the LDLR. EGCG markedly reduced the secretion of VLDL and apoB-100 in both LDLR-deficient mice (Fig. 7B) and apoB-100$^{+/+}$ controls (Fig. 5A).

Effect of EGCG on plasma lipids and lipoproteins in mice

To assess the effects on plasma lipids, we administrated EGCG intraperitoneally (38) to human apoB-100 transgenic mice. EGCG did not reduce the total plasma levels of triglycerides or cholesteryl esters. In fact, compared with the controls, EGCG-treated mice had higher mean levels of cholesterol (4.43 ± 1.8 vs. 5.47 ± 0.82 mmol/l; n = 4) and triglycerides (1.94 ± 0.35 vs. 1.22 ± 0.29 mmol/l; n =

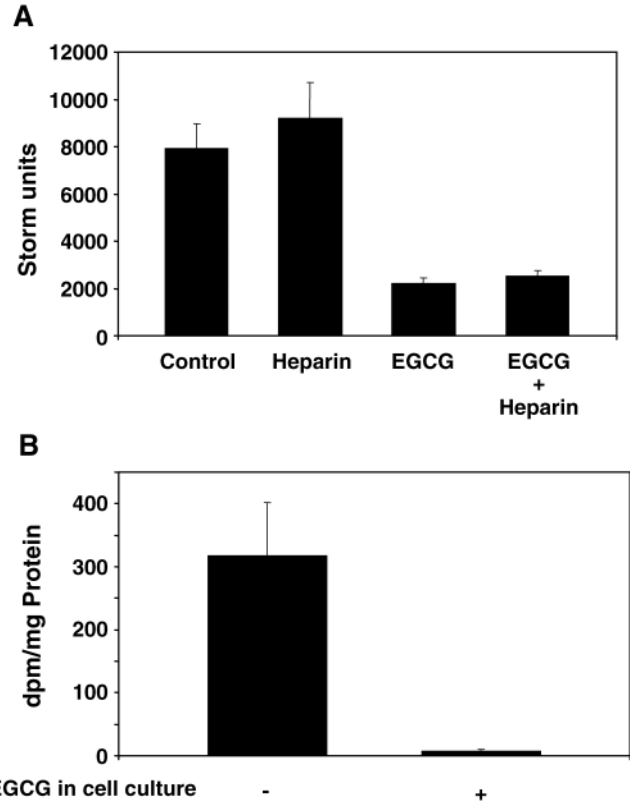

Fig. 7. Effects of heparin (A) and low density lipoprotein receptor (LDLR) deficiency (B) on the influence of EGCG on the secretion of apoB-100 VLDL. A: McA-RH7777 cells were cultured for 2 h in the presence or absence of 50 μM EGCG, pulse-labeled with [^{35}S]methionine/cysteine for 15 min, and chased for 180 min in the presence or absence of EGCG or heparin (10 mg/ml) or both. VLDL was recovered from the medium by gradient ultracentrifugation, and apoB-100 was recovered from VLDL by immunoprecipitation and SDS-PAGE. Radioactivity was determined with a phosphorimager. Values are means ± SD (n = 3). B: Hepatocytes from LDLR-deficient mice expressing human apoB-100 that had received daily intraperitoneal injections of EGCG for 1 week were cultured in the presence or absence of EGCG for 8 h, labeled with [^{35}S]methionine/cysteine for 2 h, and chased for 3 h in the presence or absence of EGCG. The radioactivity of apoB-100 in the VLDL density range was then determined. Values are means ± SD (n = 3).

4), although the differences were not statistically significant (Mann-Whitney rank-sum test).

DISCUSSION

This study shows that EGCG can increase the assembly of cytosolic lipid droplets by promoting fusion between these droplets. In addition, it demonstrates that this increase is coupled to a decrease in the assembly and secretion of VLDL attributable to sequestering of triglycerides from the assembly pathway.

The increase in the assembly of cytosolic lipid droplets was not coupled to an increase in the biosynthesis of triglycerides or to a change in the oxidation of fatty acids [the latter being consistent with findings in HepG2 cells (22)]. Thus, it can be concluded that EGCG promotes an increase in the amount of cytosolic lipid droplets that is independent of the rate of triglyceride biosynthesis. We recently (10) presented results demonstrating that lipid droplets increase in size by a fusion process that is independent of this biosynthesis. Therefore, we investigated whether EGCG might influence this process using the cell and cell-free systems recently described (10). Results from both of these experimental systems indicate that this is indeed the case. Moreover, we confirmed these results by counting the fusion events during time-lapse studies in living cells. These observations indicate that the fusion between cytosolic droplets can be regulated, which in turn means that there are ways of regulating the assembly of lipid droplets other than by just modulating the rate of triglyceride biosynthesis and degradation. We believe that EGCG may turn out to be an important tool for elucidating the mechanism behind the assembly of cytosolic droplets and the regulation of this assembly.

Results using other types of cells (21) have demonstrated that EGCG increases PLD activity. We recently showed that PLD is essential for the assembly of lipid droplets in a cell-free system (9) and in intact cells (L. Andersson, P. Boström, M. Rutberg, J. Ericsson, D. Marchesan, B. Magnusson, M. Ruiz, L. Asp, M. A. Frohman, J. Borén, and S-O. Olofsson, unpublished data). Inhibition of PLD activity reduced the formation of cytosolic lipid droplets, whereas increased activity enhanced droplet formation (9; L. Andersson, P. Boström, M. Rutberg, J. Ericsson, D. Marchesan, B. Magnusson, M. Ruiz, L. Asp, M. A. Frohman, J. Borén, and S-O. Olofsson, unpublished data). We failed to observe any effect of EGCG on PLD activity in NIH 3T3 or McA-RH7777 cells, however, or on the formation of PA. Thus, EGCG does not appear to influence the accumulation of lipid droplets via the activation of PLD in the cell types investigated.

It is well known that the fatty acids incorporated into the VLDL triglycerides are derived from cytosolic lipid droplets (4–6). This raised the question of whether an increase in cytosolic lipid droplets is always linked to an increase in the secretion of VLDL or whether triglycerides can be diverted from VLDL assembly to be stored in the cell. Therefore, we investigated whether EGCG might have an influence on the assembly and secretion of VLDL. EGCG substantially reduced apoB-100 secretion in McA-RH7777 cells and in primary mouse hepatocytes. A decreased availability of triglycerides results in an increased proteasomal degradation of apoB-100 (18, 30–41). In accordance with this, we observed that apoB-100 was lost early in the secretory pathway in cells treated with EGCG. Because EGCG did not affect other secretory proteins (i.e., transferrin, albumin, or total proteins), the influence of EGCG seems to be selective for apoB-100. Increased proteasomal degradation of apoB-100 was also supported by the observation of increased ubiquitination of apoB-100 in cells treated with EGCG. Increased proteasomal degradation of apoB-100 could also be the result of an inhibition of MTP (18, 39–41); however, we could not detect any inhibition of the triglyceride transfer activity in the presence of EGCG.

However, the lipidation of apoB-100 is complicated (11–15), starting with the cotranslational formation of a primordial lipoprotein, a process that is dependent on MTP (11, 13–15). This primordial lipoprotein needs to be converted to VLDL2 or VLDL1 to be secreted to any significant extent (17; see also 15, 16 for reviews). Because this conversion involves the addition of triglycerides to the primordial particle, it will most likely be influenced by the EGCG-induced diversion of triglyceride from the VLDL assembly pathway. Thus, it is likely that treatment with EGCG not only influences the early proteasomal degradation but also the post-endoplasmic reticulum presecretory proteolysis (18). This may be the reason why the proteasomal inhibitor lactacystin failed to completely restore the decreased secretion of apoB-100 induced by the EGCG treatment.

Because green tea induces expression of the LDLR (33), which is important in the regulation of VLDL secretion (34–37), it is possible that the effect of EGCG on the secretion of VLDLs is mediated by this receptor. However, heparin displacement studies and investigations in primary hepatocytes from LDLR-deficient mice demonstrated that the LDLR was not involved.

Together, our results indicate that EGCG promotes the formation of cytosolic lipid droplets, thereby depleting the secretory pathway of triglycerides. This results in insufficient lipidation of apoB-100 and a subsequent increased proteasomal as well as nonproteasomal degradation of the protein.

There was no significant effect of treatment with EGCG on plasma lipids. The reason for this is unclear. One possibility is that the levels of EGCG that reach the liver are too low; however, we used EGCG at concentrations similar to those used by other investigators (22, 38). Another possibility is that the metabolism of the secreted VLDLs has a greater influence on the plasma levels of triglycerides and cholesterol than does the actual secretion of VLDL, bearing in mind that the VLDL levels in mice are relatively low. These results indicate that EGCG may not be useful in lipid-lowering therapy. It should also be kept in mind that EGCG promotes the accumulation of cytosolic lipid droplets in cells very strongly. Thus, it cannot be ruled

out that EGCG may promote steatosis in organs such as the liver.

It is, of course, difficult to compare the results of an experimental study with studies in which green tea was administered orally. Because only ~0.002% of an intragastric dose of EGCG accumulates in the liver (42), ~0.001 mg of the 20–30 mg of EGCG in a cup of tea (200 ml) would accumulate in the liver. In contrast, hepatocytes in the in vitro studies were exposed to EGCG at a concentration of 0.022 mg/ml. Thus, tea drinking would not provide enough EGCG to influence lipid droplet formation. However, high doses of EGCG may give rise to an increased accumulation of cytosolic lipid droplets in hepatocytes.

In conclusion, EGCG increased the formation of cytosolic lipid droplets, thereby diverting triglycerides away from VLDL assembly, increasing the intracellular degradation of apoB-100 and decreasing VLDL secretion. Even so, EGCG did not affect plasma lipid levels significantly. These findings indicate that EGCG will be useful in studies of the intracellular turnover and secretion of triglycerides, but not for decreasing plasma lipids.

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