



17 β -Estradiol Inhibits PCSK9-Mediated LDLR Degradation Through GPER/PLC Activation in HepG2 Cells

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Plasma levels of PCSK9 are significantly higher in postmenopausal women. Pharmacologically increased estrogen levels have been shown to lower PCSK9 and LDL-C levels in animals and humans. The action of estrogen suggests that it has the ability to prevent PCSK9-mediated LDLR degradation in liver cells. However, little is known about how estrogen alters PCSK9-mediated LDLR degradation. Here, we report that 17 β -estradiol (β E2) reduces PCSK9-mediated LDLR degradation by a mechanism that involves activation of the G protein-coupled estrogen receptor (GPER). In cultured HepG2 cells, β E2 prevented the internalization of PCSK9, which subsequently lead to PCSK9-mediated LDLR degradation. The altered LDLR levels also resulted in an increase in LDL uptake that was not observed in the absence of PCSK9. In addition, we showed that clathrin was rapidly increased in the presence of PCSK9, and this increase was blocked by β E2 incubation, suggesting rapid recruitment of clathrin in HepG2 cells. PLC γ activation and intracellular Ca²⁺ release were both increased due to the rapid effect of estrogen. By using a GPER antagonist G15, we demonstrated that the GPER mediates the action of estrogen. Together, the data from this *in vitro* study demonstrate that estrogen can regulate LDLR levels mainly through GPER activation, which prevents PCSK9-dependent LDLR degradation in HepG2 cells.

Keywords: 17 β -estradiol, GPER activation, PCSK9, LDLR degradation, clathrin

INTRODUCTION

Estrogen plays a role in lipid metabolism in both physiological and pharmacological contexts. Because of the benefits of estrogen, as documented in the past decade, premenopausal women have experience delayed first-time myocardial infarction (1–3) and have approximately one half the risk of cardiovascular disease relative to that of men (4, 5). Estrogen replacement therapy is still controversial, and supplements with a high dose of estrogen plus progestin or estrogen alone have been associated with a high risk of developing breast cancer (6–9). A large randomized trial (10) from the Women's Health Initiative (WHI) indicates that estrogen plus progestin is not beneficial in postmenopausal women; there is early harm for coronary heart disease (CHD) as well as continuing harm for stroke and venous thromboembolic events (VTE). The WHI results are similar to those reported by others (11–13). However, estrogen administered to postmenopausal women increases LDL clearance and lowers the levels of total cholesterol (TC), LDL-C, and apolipoprotein B in plasma, showing the beneficial effects of estrogen in reducing the risk of cardiovascular disease (14–21).

Estrogen increases the LDL receptor (LDLR) expression to reduce the levels of total blood cholesterol and LDL-C through nuclear receptor ER α , which has been extensively investigated and is also known to be in the classical receptor pathway and to have a genomic effect (22–27). However, in C57BL/6J mice, estrogen-mediated prevention of vascular injury in response to carotid artery denudation is independent of ER α (28). Another finding suggests that protection from the development of early atherosclerotic lesions is dependent on estrogen but independent of ER α (29). Therefore, many studies strongly support a non-genomic effect of estrogen through its membrane receptor-related signal transduction (30–33).

It has been shown that the G protein-coupled estrogen receptor (GPER) mediates the rapid effect of estrogen via a non-genomic effect (31, 32, 41). The activation of the GPER by estrogen may upregulate LDLR expression and downregulate proprotein convertase subtilisin/kexin type 9 (PCSK9), a newly discovered negative regulator of lipid metabolism. As a risk factor for coronary artery disease, PCSK9 elevates LDL-C levels in plasma by binding to the extracellular domain of LDLR in hepatocytes to mediate LDLR endocytosis and degradation in hepatic lysosomes (34). In HepG2 cells expressing this receptor and the GPER agonist, G1 can decrease PCSK9 mRNA and protein levels so that LDLR protein expression increases on the cell membrane (41), thus suggesting its influence on the transcription of PCSK9. Studies in rats and humans found that high levels of endogenous estrogens reduce serum PCSK9, suggesting that estrogens also increase the number of LDLRs on hepatic cells through a posttranscriptional mechanism (35, 36). In human HuH7v hepatocarcinoma cells, LDLR mRNA is upregulated by β E2-conditioned medium with PCSK9, while β E2-conditioned medium without PCSK9 fails to upregulate LDLR expression. PCSK9-knockdown cells in medium without PCSK9 showed no increases in LDLR levels by β E2 (37). Therefore, these studies imply that PCSK9 is essential in the estrogen-induced upregulation of LDLR.

It has recently been reported that the GPER is a receptor in the plasma membrane that can be translocated to the endoplasmic reticulum (ER) (30). In HepG2 cells, β E2 is shown to be rapidly internalized via clathrin-coated vesicles and transported to sorting endosomes (38). Therefore, the aim of this study was to investigate the mechanism by which β E2 modulates PCSK9-mediated LDLR degradation and the role of clathrin in GPER activation by β E2. Our data showed that GPER activation by β E2 is dependent on phospholipase C- γ (PLC γ) and intracellular Ca²⁺ signaling, which alters the distribution of clathrin, thereby inhibiting PCSK9-mediated degradation of LDLR. These data may help understand the molecular mechanisms underlying the physiological effects of estrogens.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant human PCSK9 (rhPCSK9, gl-10916) was purchased from Genlocus (Chengdu, China). β E2 (E2758-1G) was purchased from Sigma-Aldrich (Shanghai, China). The GPER antagonist G15 (1161002-05-6) was purchased

from Cayman Chemical (Michigan, USA). The PLC inhibitor U73122 (U6756-5 MG) was purchased from Sigma-Aldrich (San Francisco, USA). Anti-LDLR antibody (sc-373830) was ordered from Santa Cruz (Dallas, USA). Anti-phospho-PLC γ (Tyr783) polyclonal antibody (2821) and anti-PLC γ rabbit antibody (2822) were purchased from Cell Signaling (Hongkong, China). Anti-clathrin rabbit polyclonal antibody (ab59710) was purchased from Abcam (Shanghai, China). The BODIPYTM FL LDL (L3483), Alexa Fluor[®] 488 protein labeling kit (A10235), Alexa Fluor[®] 488-conjugated goat anti-mouse IgG (A11001) and Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG (A11008) were all purchased from Thermo Fisher (Shanghai, China). A cAMP parameter assay kit (KGE002B) was purchased from R&D (Minneapolis, USA). The PrimeScriptTM II 1st Strand cDNA synthesis kit (6210A) and TB GreenTM Premix Ex Taq II (RR420B) were purchased from TaKaRa (Dalian, China).

Cell Culture

HepG2 cells (ATCC, USA) were maintained at 37°C in phenol red-free DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin and allowed to grow until 85% confluent prior to the experiment.

PCSK9 Internalization Assay

For the internalization assay, rhPCSK9 was labeled with an Alexa Fluor[®] 488 from the protein labeling kit (^{AF}-PCSK9). To determine whether β E2 blocked the internalization of PCSK9, HepG2 cells were seeded into 48-well plates at 4×10^4 cells per well in growth medium. After serum deprivation for 16 h, the cells were incubated with 25 μ g/mL ^{AF}-PCSK9 for 0.5–6 h and treated with 0.01–10 μ M β E2 for 2 h in the presence of ^{AF}-PCSK9 (25 μ g/mL). For all assays, the cells were pretreated with 1 μ M G15 for 15 min prior to the addition of β E2 to block GPER action. After a series of wash steps with PBS, internalized ^{AF}-PCSK9 was directly observed under an inverted fluorescence microscope, and the fluorescence intensity of ^{AF}-PCSK9 in isopropyl alcohol was detected by a SpectraMax M5 reader and reported in RFUs.

LDLR Protein Assay

To investigate whether β E2 prevents PCSK9-mediated LDLR degradation, HepG2 cells were seeded into 6.2 cm²-cell imaging dish (NEST, Suzhou, China) at 6×10^4 cells per well in growth medium and allowed to adhere to the plate for 24 h. After serum deprivation for 16 h, the cells were treated with or without 0.01–1 μ M β E2 for 6 h in the presence or absence of rhPCSK9 (25 μ g/mL). The expression level of LDLR was detected by western blotting and immunofluorescence.

LDLR mRNA Assay

For detection of LDLR mRNA, HepG2 cells were seeded into 6-well plates at 1×10^5 cells per well in growth medium and allowed to adhere to the plate for 24 h. After serum deprivation for 16 h, the cells were incubated with or without 0.01–1 μ M β E2 for 6 h in the presence of rhPCSK9 (25 μ g/mL). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from 1 μ g

RNA using a PrimeScriptTM II 1st strand cDNA synthesis kit according to the manufacturer's protocol (TaKaRa). Real-time quantitative PCR was performed to detect the expression of LDLR mRNA in a CFX96 PCR system (Bio-Rad) with 1 μ L of cDNA diluted 25-fold in 12.5 μ L of the reaction mixture with TB Green Premix Ex Taq II and 0.1 μ M forward and reverse primers for LDLR and β -actin. Specific primers for LDLR and β -actin were used: for LDLR, forward, 5'-GTTATTCAGGGAGAACGGCT-3' and reverse, 5'-GAGTCAACCCAGTAGAGGC-3'; and for β -actin, forward, 5'-CTGGGCATGGAGTCCTGTG-3', and reverse, 5'-ATCTTCATTGTGCTGGGTG-3'. The primers were obtained from GENEWIZ (Suzhou, China). We set amplification parameters: 95°C for 30 s; 40 cycles of 95°C for 5 s; and 56°C for 30 s.

LDL Uptake Assay

LDL uptake was measured using a BODIPY[®] FL kit. HepG2 cells were seeded into 48-well plates at 4×10^4 cells per well in growth medium and allowed to adhere to the plate for 24 h. After serum deprivation for 16 h, the cells were treated with or without 0.01–10 μ M β E2 for 4 h in the presence of rhPCSK9 (25 μ g/mL). For the assay, the cells were pretreated with 1 μ M G15 for 15 min prior to the addition of β E2 to block GPER action. BODIPYTM FL LDL (6 μ g/ml) was added for 3 h, and then the cells were then washed with PBS. Isopropyl alcohol was added to measure the uptake of BODIPYTM FL LDL. The plates were read with a SpectraMax M5 reader (Molecular Devices, Sunnyvale, USA).

Clathrin Assay

Clathrin was also measured for the distribution assay. HepG2 cells were seeded into 6.2 cm²-cell imaging dish (NEST, Suzhou, China) at 6×10^4 cells per well in growth medium and allowed to adhere to the plate for 24 h. After serum deprivation for 16 h, the cells were incubated with 25 μ g/mL rhPCSK9 for 0–2 h and treated with 0.01–1 μ M β E2 for 2 h in the presence of rhPCSK9 (25 μ g/mL). For all assays, the cells were pretreated with 1 μ M G15 for 15 min prior to the addition of β E2 to block GPER action. After a series of wash steps with PBS, clathrin was detected by immunofluorescence with the corresponding antibodies.

Calcium Mobilization Assay

Calcium mobilization was detected by Fluo-4 AM (Invitrogen, Shanghai, China). HepG2 cells were seeded in a cell imaging dish at 6×10^4 cells per well and allowed to adhere to the plate for 24 h. After serum deprivation for 16 h, the cells were washed with Ca²⁺-free HBSS (136.8 mM NaCl, 5.4 mM KCl, 5.5 mM Glucose, 0.44 mM KH₂PO₄, and 0.33 mM Na₂HPO₄, pH 7.2) and then loaded with 5 μ M Fluo-4 AM for 20 min. The cells were washed again with Ca²⁺-free HBSS. Each cell imaging dish was placed in a laser scanning confocal microscope (LSM710, ZEISS). First, the fluorescence intensity images were collected every 15 s for 5 min, and 0.1 μ M E2 was then added and fluorescence was monitored continuously for 15 min. For the assay, the cells were treated with 1 μ M G15 and 1 μ M U73122 for 15 min prior to the addition of E2 to block GPER and PLC γ action. Data were quantified and analyzed using ZEN 2010 software. The background was

subtracted, and the fluorescence was expressed relative to values at the beginning of the experiment.

Western Blot Analysis

HepG2 cells were seeded into T25-cell culture bottles at 5×10^5 cells with 5 mL growth medium. After serum deprivation for 16 h, the cells were treated with or without 0.1 μ M β E2 for 5 min in the presence of rhPCSK9 (25 μ g/mL). For the assay, the cells were pretreated with 1 μ M G15 for 15 min prior to the addition of β E2 to block GPER action. After treated with 0.1 μ M β E2 for 5 min or 0.01–1 μ M β E2 for 6 h, the cells were washed twice with cold PBS and lysed with 1 \times RIPA buffer (Beyotime, China) supplemented with a protease inhibitor PMSF (1 mM) to obtain the total protein. An equal amount of total protein from each lysate was loaded and separated on 4%–10% gels for SDS-PAGE and electroblotted to polyvinylidene fluoride (PVDF) membranes. The primary antibodies used included anti-LDLR mouse monoclonal antibody (1:150 diluted in PBS), anti-pPLC γ (Tyr783) rabbit antibody (1:500 diluted in PBS), and anti-PLC γ rabbit antibody (1:500 diluted in PBS). The secondary HRP-conjugated antibody was goat anti-mouse IgG (1:4000 diluted in PBS) and goat anti-rabbit IgG (1:4000 diluted in PBS). Western blots were measured for chemiluminescence (Thermo Fisher), detected by ImageQuant LAS 500 (General Electric Company, Boston, USA), and analyzed by Image Plus 6.0 analysis software.

Immunofluorescence Staining

Experiments were performed in cell imaging dishes as described in the methods section LDLR Protein Assay and Clathrin Assay. HepG2 cells were washed three times with PBS and fixed with a 4% paraformaldehyde solution for 20 min. The fixed cells were permeabilized with PBS containing 0.3% Triton X-100 for 3 min at 4°C and washed three times with PBS containing 0.5% BSA. The cells were incubated with 3% BSA in PBS for 30 min at 37°C and then incubated with primary antibodies: anti-LDLR mouse monoclonal antibody (1:100 diluted in PBS) and anti-clathrin rabbit polyclonal antibody (1:500 diluted in PBS) at 4°C overnight. Antibodies were stained with Alexa Fluor[®] 488-conjugated goat anti-mouse IgG (1:250 diluted in PBS) and Fluor[®] 488-conjugated goat anti-rabbit IgG (1:250 diluted in PBS) at room temperature for 1 h. Excess antibody was removed by washing the cells with PBS, and the cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and then viewed with a confocal microscope (LSM710, Carl Zeiss AG, German).

Statistical Analysis

Data are presented as the mean \pm SEM. Differences among groups were tested by SPSS 17.0 software with one-way ANOVAs followed by an LSD or Duncan test. Comparisons were considered to be statistically significant if $P < 0.05$.

RESULTS

β -Estradiol Blocked PCSK9 Internalization

PCSK9 is a secreted protein and negative regulator of LDLR in hepatocytes. It binds to LDLR at the cell surface and can mediate LDLR into lysosomes, where it is degraded. We first examined

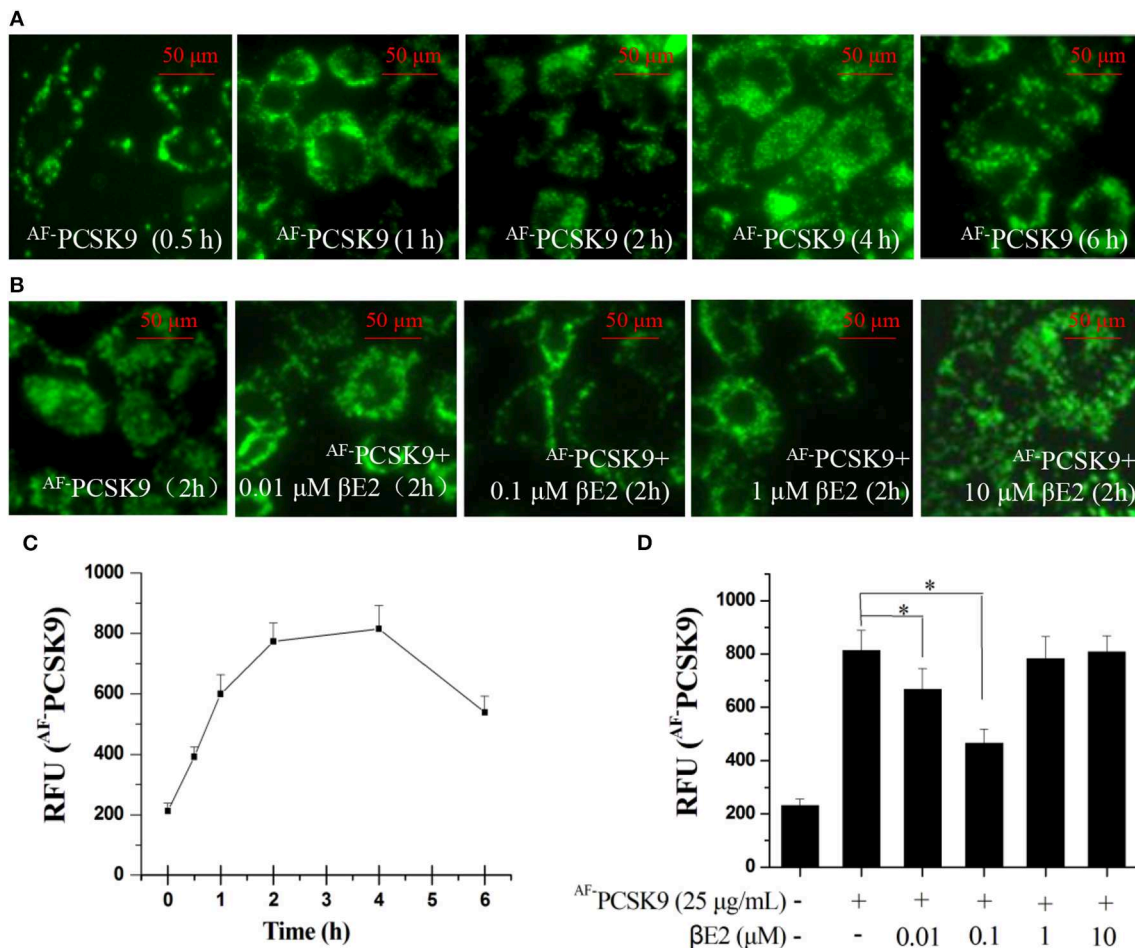


FIGURE 1 | β E2 blocked the internalization of PCSK9. **(A)** Alexa Fluor 488 dye-labeled PCSK9 (25 μ g/mL) was added to HepG2 cells not treated with β E2. AF -PCSK9 internalization was observed at specified time intervals. **(B)** Internalization of AF -PCSK9 was blocked at the cell surface by β E2 administered at specified concentration ranges. **(C,D)** Quantification of AF -PCSK9. The relative fluorescent unit (RFU) of AF -PCSK9 was determined by ZEISS 2010 software. Values represent the means \pm SEM, $n = 3$; * $P < 0.05$ for a comparison between two groups.

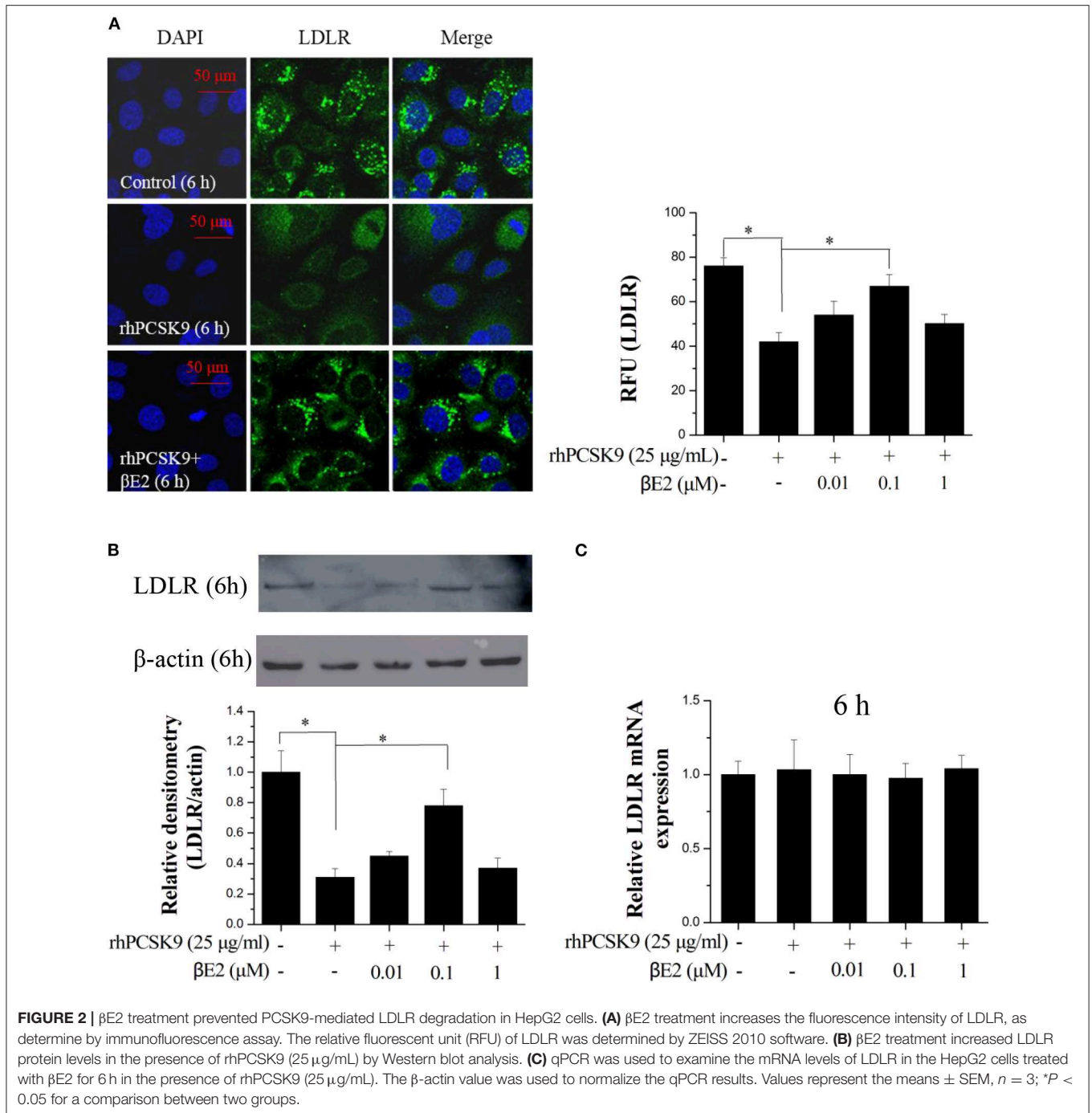
PCSK9 internalization using an Alexa Fluor 488 dye-labeled rhPCSK9 (AF -PCSK9) in HepG2 cells expressing LDLR. The fluorescence signals were detectable in the HepG2 cells at 30 min and peaked at 2 and 4 h after AF -PCSK9 (25 μ g/mL) was added to the cells. The localization of AF -PCSK9 was initially at the cell surface, and it was later localized throughout the cytoplasm (**Figure 1A**) with increasing quantity (**Figure 1C**), indicating the internalization and intracellular trafficking of AF -PCSK9.

To determine whether β E2 blocked the internalization of PCSK9, HepG2 cells were incubated with 0.01–10 μ M β E2 for 2 h in the presence of AF -PCSK9. In the HepG2 cells without β E2, the fluorescence signals were distributed throughout the cytoplasm after AF -PCSK9 (25 μ g/mL) was added. In contrast, β E2 treatment decreased the cytoplasmic distribution of AF -PCSK9 and increased the distribution of AF -PCSK9 at the cell surface (**Figure 1B**), and this effect was most pronounced with the β E2 treatment of 0.1 μ M (**Figure 1D**). These results indicate that estrogen can block the internalization of PCSK9

and thus may prevent PCSK9-mediated LDLR degradation. In cultured HepG2 cells, a high concentration of β E2 (10 μ M) led to significant cytotoxicity. Therefore, all subsequent experiments were performed with 0.01–1 μ M β E2 treatments.

β E2 Prevented PCSK9-Mediated LDLR Degradation

As expected, the immunofluorescence from the stained LDLR was significantly enhanced after treatment with β E2 at 0.1 μ M but not 1 μ M for 6 h compared with that in the control HepG2 cells that were treated only with PCSK9 (**Figure 2A**). A similar result was obtained using Western blotting (**Figure 2B**). β E2 treatment at 0.1 μ M significantly increased LDLR levels in the presence of rhPCSK9 (25 μ g/mL), and β E2 treatment at 1 μ M did not increase LDLR levels. In addition, LDLR mRNA levels were detected by qPCR, and no change was observed following β E2 treatment (**Figure 2C**). The addition of rhPCSK9 to the HepG2 cells for 6 h resulted in the reduction in LDLR levels, indicating



that PCSK9 mediated LDLR degradation. These data suggest that β E2 treatment at 0.1 μ M can inhibit PCSK9-mediated LDLR degradation in HepG2 cells.

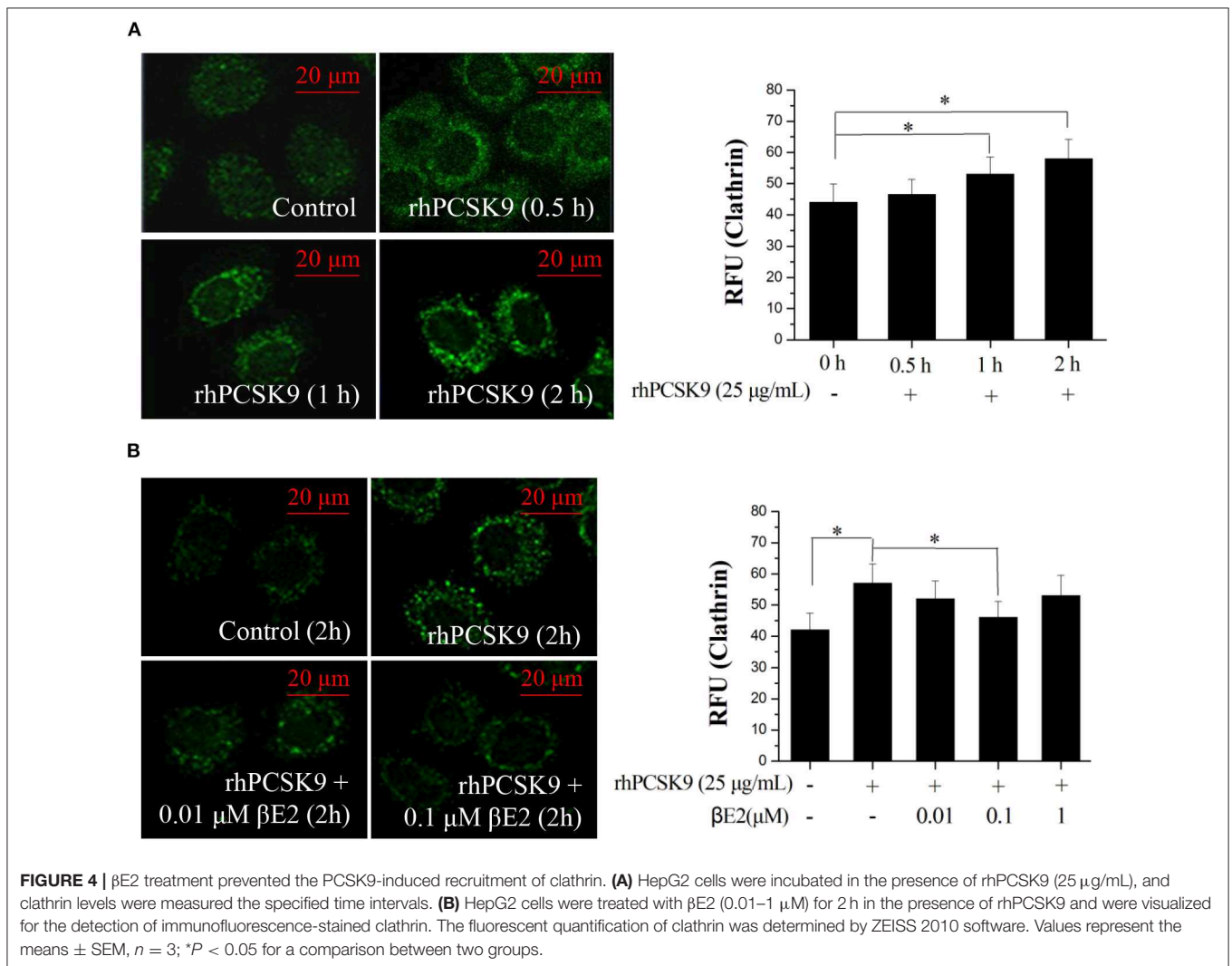
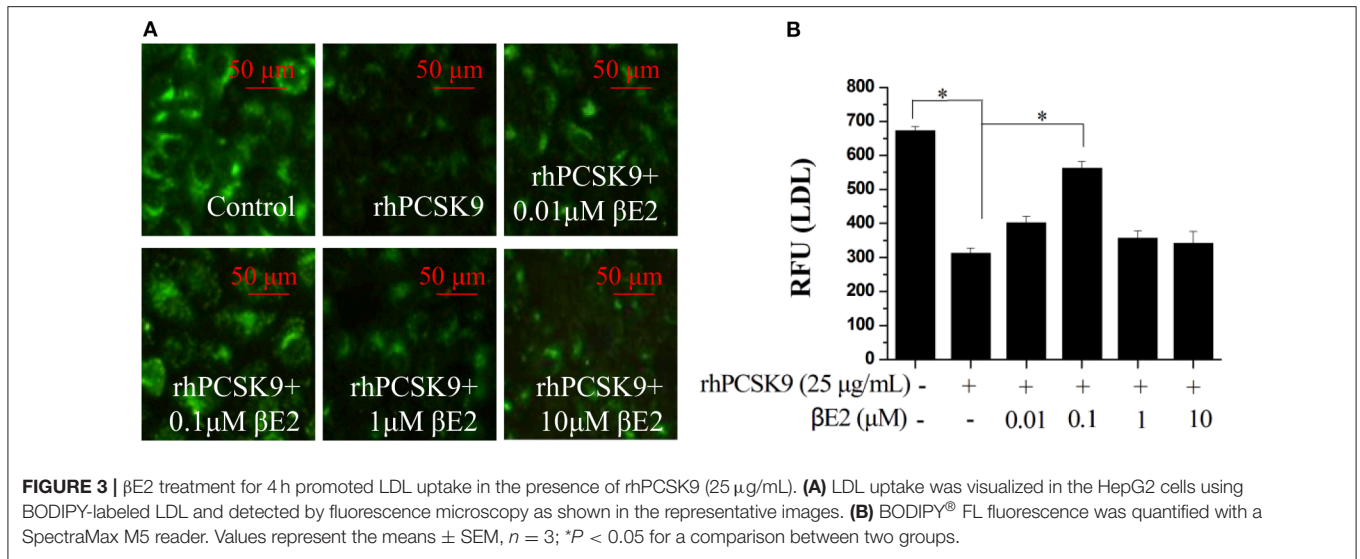
β E2 Modulates Cellular LDL Uptake in the Presence of PCSK9

The addition of recombinant PCSK9 resulted in LDLR degradation. The inhibitory effect of β E2 on PCSK9-mediated LDLR degradation may promote the uptake of LDL-C into cells. As shown in **Figure 3**, β E2 treatment increased the fluorescence

intensity of the labeled LDL in the HepG2 cells compared to the intensity observed with rhPCSK9 treatment alone, indicating that β E2 promotes LDL uptake into cells in the presence of rhPCSK9. Consistent with the levels of LDLR protein expression, β E2 treatment at 1 μ M did not promote LDL uptake.

β E2 Prevents PCSK9-Induced Increases in Clathrin

Previous studies have suggested that clathrin trafficking is involved in PCSK9-mediated LDLR degradation in hepatic cells.



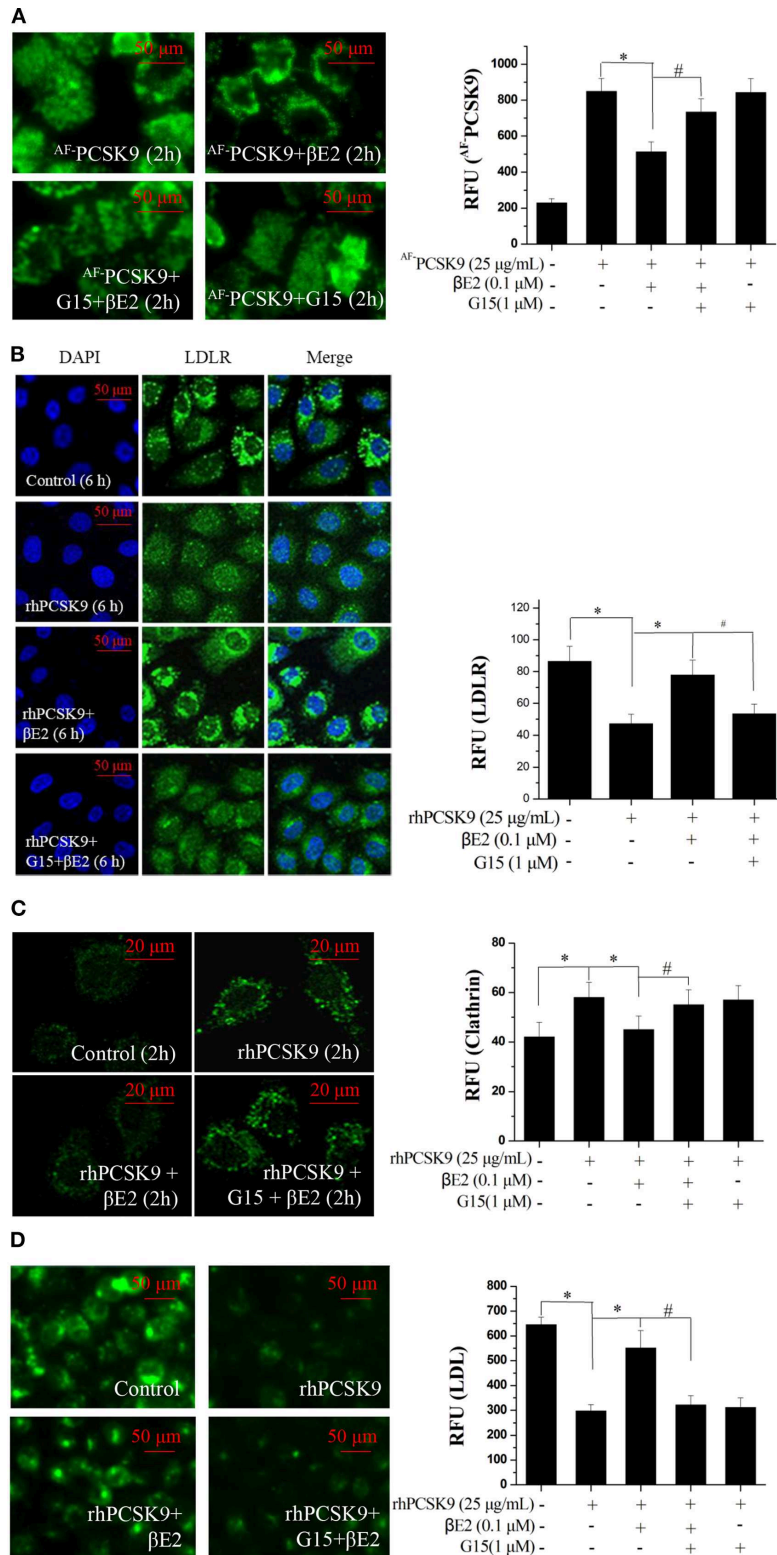
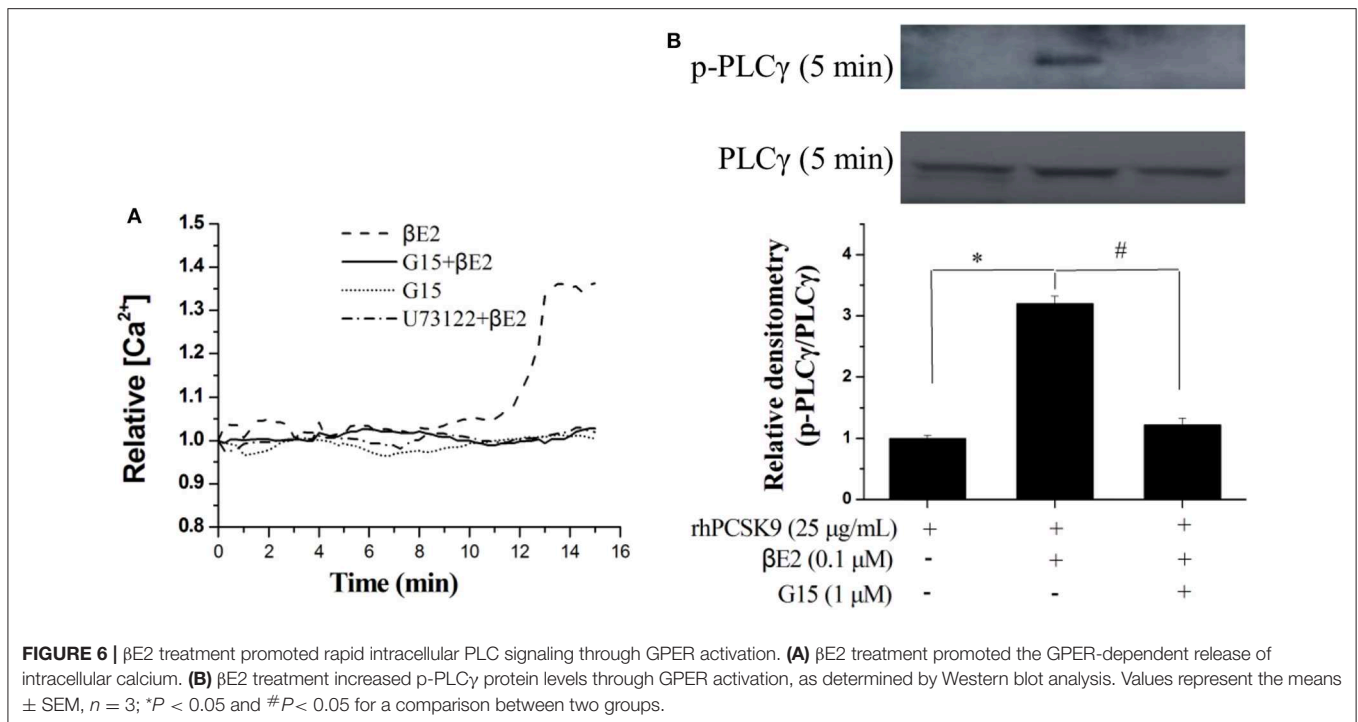


FIGURE 5 | β E2 treatment prevented the PCSK9-mediated LDLR degradation by activating GPR30. **(A)** β E2-treated cells for 4 h with and without G15 were visualized for the internalization of AF -PCSK9 (25 μ g/mL) by fluorescence microscopy. **(B,C)** Immunofluorescence staining of LDLR and clathrin, and the intensity of which was quantified in β E2-treated cells treated with and without G15, respectively. **(D)** After 4 h, the cells were exposed to BODIPY-labeled LDL, and then, the uptake was visualized through fluorescence microscopy. Fluorescence intensity was quantified with a SpectraMax M5 reader. Values represent the means \pm SEM, $n = 3$, * $P < 0.05$ and # $P < 0.05$ for comparisons between two groups.



The major route of PCSK9-dependent LDLR degradation in HepG2 cells has been demonstrated to be through clathrin-mediated endocytosis (39). Knocking down the clathrin heavy chain increased the LDLR protein levels in HuH7 cells (40), indicating that clathrin is involved in PCSK9-mediated LDLR degradation in hepatocytes. We asked whether clathrin trafficking pathway might also be affected by β E2. HepG2 cells were incubated with rhPCSK9 for 0–2 h and then examined for clathrin distribution by staining an antibody to clathrin. The addition of rhPCSK9 caused an increase in clathrin stain intensity in HepG2 cells, with large cytoplasmic and perinuclear localization peaks at 2 h, in contrast to the weak staining observed in the absence of rhPCSK9 (Figure 4A). In parallel experiments, clathrin staining was decreased by β E2 treatment (Figure 4B). These results suggest that PCSK9 may induce an increase in clathrin levels. It is very likely that clathrin is recruited by the addition of rhPCSK9 and then participates in PCSK9-mediated LDLR degradation in HepG2 cells. β E2 may prevent clathrin-dependent LDLR endocytosis, thereby inhibiting PCSK9-mediated LDLR degradation.

β E2 Prevents PCSK9-Mediated LDLR Degradation by Activating the GPER

The GPER may function uniquely as an intracellular transmembrane receptor to contribute to normal estrogen physiology as well as pathophysiology (30). In particular, GPER activation has been shown to upregulate LDLR expression and consequently LDL metabolism (41). To determine whether the effect of β E2 was mediated through the GPER, HepG2 cells were pretreated with G15, a selective GPER

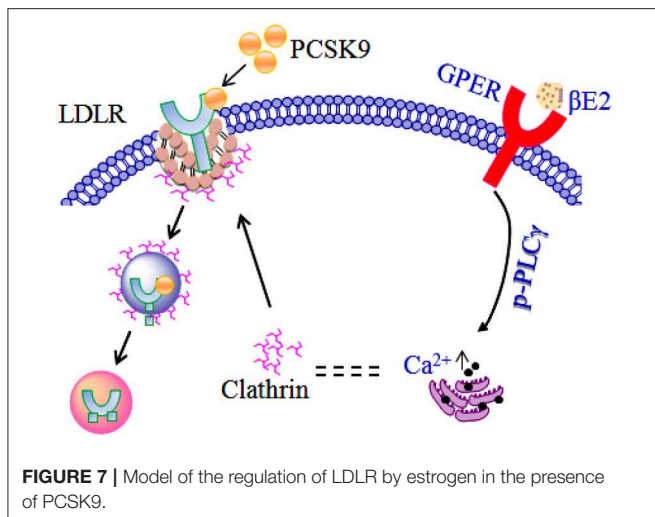
antagonist, followed by 0.1 μ M β E2 treatment for 2 h in the presence of ^{AF}-PCSK9. As shown in Figure 5A, ^{AF}-PCSK9 was mainly distributed at the cell surface after 0.1 μ M β E2 treatment for 2 h. However, G15 pretreatment for 15 min increased the distribution of ^{AF}-PCSK9 in the cytoplasm, exhibiting a similar distribution as that of the ^{AF}-PCSK9 to control cells not treated with β E2. G15 pretreatment also inhibited the effects of β E2, increasing LDLR degradation (Figure 5B) and clathrin levels (Figure 5C). In addition, β E2-enhanced LDL uptake was prevented by G15 pretreatment (Figure 5D).

β E2 Promotes Rapid Intracellular PLC Signaling Through GPER Activation

The GPER is involved in intracellular calcium mobilization in different cell types, and its role is related to the rapid activation of phospholipase C. We next investigated the rapid effect of β E2 on GPER activation by measuring the release of intracellular calcium. Intracellular Ca²⁺ was released from in HepG2 cells after 15 min of 0.1 μ M β E2. The effects of β E2 can be blocked by G15 and U73122 (PLC inhibitor) pretreatment of cells (Figure 6A) for 15 min. Phospholipase C- γ (PLC γ), in the Ca²⁺ signaling pathway, was also activated after a 5 min β E2 incubation. The effect of β E2 can be blocked by G15 pretreatment (Figure 6B), indicating the rapid activation of PLC γ through GPER stimulation.

DISCUSSION

Several studies have been reported to suggest that estrogen regulates lipoprotein metabolism through the GPER in human



hepatic HepG2 cells or in HuH7 cells (37). However, no detectable change in PCSK9 transcription levels was found with increased β E2 doses (37). β E2 may upregulate LDLR through extracellular PCSK9-dependent mechanisms in human hepatic cells (37). The results presented here provide data in support of this hypothesis, i.e., that the effect of β E2 regulation of LDLR is dependent upon extracellular PCSK9. There was a significant increase in LDLR protein expression upon β E2 treatment in the presence of rhPCSK9. Moreover, β E2 inhibits PCSK9-mediated LDLR degradation, which results in the increase of LDL uptake by HepG2 cells, an effect that was blocked by G15, a GPER antagonist.

It has been shown that PCSK9 binds to the extracellular domain of LDLR to trigger LDLR degradation in lysosomes (39, 42, 43). Clathrin is a cytosolic protein, and the importance of the clathrin heavy chain has been demonstrated through the PCSK9-dependent degradation of LDLR (39). Although there is no evidence that clathrin recruitment is affected by PCSK9, the HepG2 cells treated with rhPCSK9 had increased levels of clathrin in the cytoplasm and perinucleus. It is likely that PCSK9 rapidly binds to LDLR to form a PCSK9-LDLR complex that recruits clathrin, and the complex then enters cells by a clathrin-dependent endocytosis pathway that leads to LDLR degradation. β E2 may prevent clathrin-dependent LDLR endocytosis, thereby inhibiting PCSK9-mediated LDLR degradation, which was blocked by G15. Therefore, β E2 increases LDLR protein expression, likely via GPER activation.

GPER also contributes to the activation of the phospholipase C (PLC) pathway in other cell types (44). The activation of PLC may lead to an increase in intracellular Ca^{2+} and regulated clathrin-dependent endocytosis (45, 46). In HepG2 cells, PLC-gamma (PLC γ) was activated, and intracellular Ca^{2+} release was detected after β E2 treatment. These effects of β E2 were

blocked by the GPER antagonist G15 and the PLC inhibitor U73122, indicating a role for estrogen in the rapid regulation of LDLR lysosomal degradation through GPER. The regulation of PCSK9 levels has been shown to be cAMP-dependent (47), and cAMP is another messenger pathway known to be regulated by GPER activation. We also demonstrate that β E2 rapidly stimulates cAMP production which was blocked by G15 (data not shown).

Figure 7 presents a schematic diagram summarizing our results. PCSK9 binds to the extracellular domain of LDLR to mediate LDLR endocytosis and degradation in a clathrin-dependent manner. The molecular mechanism of estrogen inhibition of PCSK9-mediated LDLR degradation can be explained: activating GPER in the membrane by β E2 (48) rapidly initiates the phosphorylation of PLC γ , leading to intracellular Ca^{2+} release, which in turn alters clathrin distribution or influences the clathrin trafficking pathway to affect the internalization of the PCSK9-LDLR complex. Another possible explanation is that the GPER is localized to the endoplasmic reticulum (30), where the binding of β E2 initiates activation of PLC γ and recruits clathrin assembly proteins to it, forming a clathrin-coated pit (38). The coated pit is internalized and prevents clathrin-dependent LDLR endocytosis, thereby inhibiting PCSK9-mediated LDLR degradation. The GPER may function uniquely as an intracellular transmembrane receptor that contributes to normal estrogen physiology as well as pathophysiology (30). Investigations into other possible intracellular GPER isoforms activated by estrogen would be of great interest.

In summary, our results highlight the important role of estrogen in regulating LDLR levels through GPER activation and being associated with clathrin trafficking pathway. Therefore, estrogen may have a role in modulating the levels of total blood cholesterol and LDL-C in hypercholesterolemic women.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

WF designed and planned the study and performed the experiments. X-PG and L-MY designed and planned the study and wrote the manuscript. SZ, Y-PD, and W-JZ performed the experiments and analyzed the data.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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