The steroidal analog GW707 activates the SREBP pathway through disruption of intracellular cholesterol trafficking

Jessie Zhang,* Nicole Dudley-Rucker,* Jan R. Crowley,† Elvira Lopez-Perez,§ Marc Issandou,§ Jean E. Schaffer,*, and Daniel S. Ory[1],*,††**

Center for Cardiovascular Research,* Department of Internal Medicine, and Mass Spectrometry Facility,† Department of Internal Medicine, Laboratoires GlaxoSmithKline,§ 91951 Les Ulis cedex, France; and Departments of Molecular Biology and Pharmacology** and Cell Biology and Physiology,†† Washington University School of Medicine, St. Louis, MO 63110-1010

Abstract **Recently, a new class of lipid-lowering agents has been described that upregulate LDL receptor (LDLr) activity. These agents are proposed to activate sterol-regulated gene expression through binding to the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP). Here, we show that the steroidal LDLr upregulator, GW707, induces accumulation of lysosomal free cholesterol and inhibits LDL-stimulated cholesterol esterification, similar to that observed in U18666A-treated cells and in Niemann-Pick type C1 (NPC1) mutants. Moreover, we demonstrate that induction of the NPC-like phenotype by GW707 is independent of SCAP function. We find that treatment with GW707 does not increase SREBP-dependent gene expression above that observed in lipoprotein-starved cells. Rather, we show that the apparent increase in SREBP-dependent activity in GW707-treated cells is attributable to a failure to appropriately suppress sterol-regulated gene expression, as has been shown previously for U18666A-treated cells and NPC mutant fibroblasts. We further demonstrate that cells treated with either GW707 or U18666A fail to appropriately generate 27-hydroxycholesterol in response to LDL cholesterol. Taken together, these findings support a mechanism in which GW707 exerts its hypolipidemic effects through disruption of late endosomal/lysosomal sterol trafficking and subsequent stimulation of LDLr activity.**—Zhang, J., N. Dudley-Rucker, J. R. Crowley, E. Lopez-Perez, M. Issandou, J. E. Schaffer, and D. S. Ory. **The steroidal analog GW707 activates the SREBP pathway through disruption of intracellular cholesterol trafficking.** ***J. Lipid Res.*** **2004.** 45: **223–231.**

Supplementary key words sterol regulatory element binding protein • cleavage-activating protein ligands • Niemann-Pick type C • sterol-sensing domain • 27-hydroxycholesterol

Hypercholesterolemia is a major risk factor for the development of coronary artery and cerebral vascular disease. Increased plasma levels of LDL lead to deposition of excess cholesterol in arteries, initiating atherosclerosis. Treatment with 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) lowers plasma LDL and decreases cardiovascular mortality. The beneficial effects of statin therapy primarily result from the depletion of cellular cholesterol and the increased expression of the LDL receptor (LDLr), thereby promoting clearance of LDL particles and reducing plasma LDL (1, 2). Recently, a novel class of potent cholesterol-lowering agents has been described that upregulates LDLr activity, leading to marked reduction of both LDL cholesterol and triglycerides in vivo (3). These cholesterol-lowering agents, initially designated SCAP ligands and referred to here as LDLr upregulators, were identified in a screen for transcriptional activators of the human LDLr promoter and are proposed to bind to the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP) through direct interaction with the sterol-sensing domain of SCAP.

The steroidal LDLr upregulator GW707 is structurally similar to the amphiphilic compound 3-β-[2-(diethylamino) methoxy]androst-5-en-17-one (U18666A) (**Fig. 1**), which is known to interfere with intracellular sterol trafficking (4). Both compounds consist of a planar sterol nucleus with an ether linkage at the C-3 position and an alkyl side chain that contains a tertiary amine. U18666A differs from GW707 in that the sterol side chain is absent from the C-17 position. Cells treated with U18666A accumulate lysosomal free cholesterol and demonstrate impaired movement of

Abbreviations: ER, endoplasmic reticulum; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; LDLr, LDL receptor; LPDS, lipoprotein-deficient serum; LXR, liver X receptor; NPC, Niemann-Pick type C; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

[1] To whom correspondence should be addressed.
e-mail: dory@wustl.edu

Manuscript received 26 September 2003 and in revised form 31 October 2003.

Published, JLR Papers in Press, November 16, 2003.
DOI 10.1194/jlr.M300409-JLR200

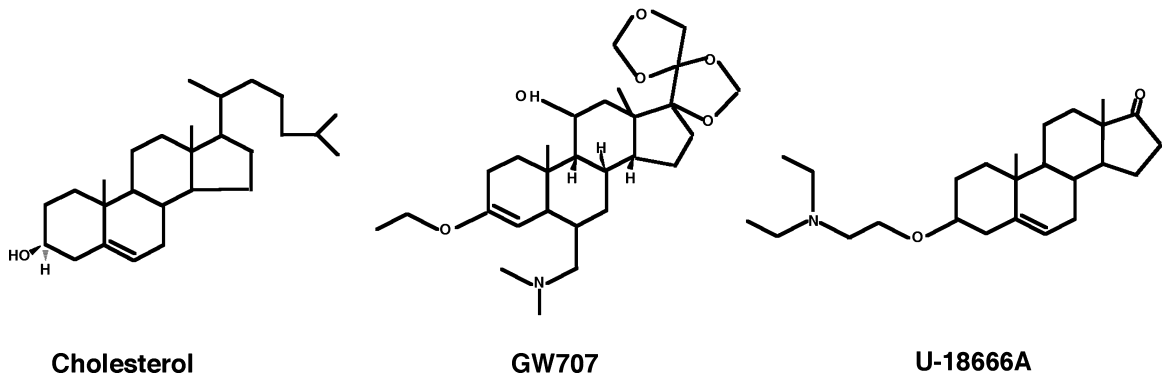

Fig. 1. Structures of the GW707 and U18666A steroidal analogs. Both GW707 and U18666A possess an ether linkage at the C-3 position and alkyl side chain attached to the steroid A-ring that contains a tertiary amine. GW707 has two heterocyclic spiro rings at the C-17 position, whereas in U18666A the sterol side chain is absent.

cholesterol both to and from the plasma membrane (4–8). Treatment of cells with U18666A also inhibits LDL-stimulated cholesterol esterification and prevents the suppression of sterol-regulated gene expression (4).

The sterol-trafficking defects induced by U18666A phenocopy the cellular lesion in the Niemann-Pick type C (NPC) disease, a cholesterol storage disorder characterized by cholesterol accumulation in the liver, spleen, and central nervous system (9). The major NPC disease gene, NPC1, encodes a late endosomal polytopic protein with a conserved domain that shares sequence homology with the sterol-sensing domains of HMG-CoA reductase and SCAP (10, 11). A second disease locus encodes NPC2, a 132 amino acid soluble lysosomal protein that has been shown to specifically bind cholesterol with a 1:1 stoichiometry and submicromolar affinity (12–15). Cells harboring mutations in NPC1 and NPC2 accumulate free cholesterol in an aberrant lysosomal organelle and have impaired rates of esterification of LDL cholesterol (16, 17). Despite increased total cellular cholesterol, fibroblasts from NPC1 and NPC2 patients are unable to suppress LDLr activity and de novo cholesterol synthesis (18–20). Recently, we have shown that the sterol regulatory defects in both NPC1 and NPC2 mutants result from the failure of LDL cholesterol to both suppress SREBP-dependent gene expression and promote liver X receptor (LXR)-mediated responses (20).

Although the mechanism of action of U18666A is not well understood, the cellular phenotype induced by this compound suggests that it disrupts trafficking of LDL cholesterol through the NPC pathway (4, 6). We hypothesized that the steroidal compound GW707 similarly may act through inhibition of either NPC1 or NPC2 function. Therefore, the lipid-lowering effects of LDLr upregulators may result from disruption of intracellular sterol-trafficking pathways and downstream sterol-regulated gene expression, rather than through the direct activation of SCAP. In this study, we show that GW707, like the amphiphilic compound U18666A, induces an NPC-like phenotype in Chinese hamster ovary (CHO) cells and that induction of this phenotype is independent of SCAP function. Rather than upregulating SREBP-dependent gene expression, we show that the increased sterol-regulated activity in GW707-treated cells is attributable to a failure to appropriately suppress SREBP-dependent gene expression, similar to that observed in NPC mutant fibroblasts. Our findings support a mechanism in which GW707 exerts its lipid-lowering effects through inhibition of the late endosomal/lysosomal sterol-trafficking pathway. Moreover, GW707 may serve as a useful tool to probe pathways of intracellular cholesterol trafficking.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, glutamine, and penicillin/streptomycin were obtained from Invitrogen. Lipoprotein-deficient serum (LPDS) was obtained from Cocalico Labs. U18666A was obtained from Biomol. Steroidal compound GW707 was provided by GSK. Oleic acid and cholesteryl oleate were obtained from Nu-Check Prep. [^{3}H]Cholesterol (75 Ci/mmol), [9,10-^{3}H]oleic acid (5 Ci/mmol), and [oleate-1-^{14}C]cholesteryl oleate (59.5 mCi/mmol) were obtained from Perkin-Elmer Life Sciences Products. 27-Hydroxycholesterol (27-HC) was obtained from Research Plus, Inc. Compactin, fetal bovine serum (FBS), filipin complex, human LDL, and mevalonic acid were obtained from Sigma. Cholesterol, 24(*S*)-hydroxycholesterol (24-HC), and 25-hydroxycholesterol (25-HC) were obtained from Steraloids, Inc.

Plasmids

The LDLp-588luc construct used in the reporter assays contains the human LDLr promoter linked to a luciferase reporter (3). The thymidine kinase (TK) promoter-*Renilla* luciferase construct, pRL-TK, was from Promega.

Cell lines

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC; CRL-9618). M12 cells are a mutant CHO-K1 cell line with a deletion of the NPC1 locus (7). The CHO/NPC1-28 cell line expressing human NPC1 was generated as previously described (7). 25-RA cells were provided by T. Y. Chang (Dartmouth College) (21). SRD-13A cells were a gift from M. Brown (22). Normal skin fibroblasts (CRL-1474) were obtained from

ATCC. The NPC1-null mutant human skin fibroblast cell line NPC1^{1628delC} (NIH 98.016) was provided by P. Pentchev (National Institutes of Health) (23). The NPC2-null mutant human skin fibroblast cell line NPC2$^{G20\times}$ (NIH 99.04) was provided by A. Fensom (United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, London, UK) (24). The use of human fibroblast cell lines was approved by the Human Studies Committee at the Washington University Medical Center.

Cell culture

Cells were maintained in monolayer culture at 37°C with 5% CO_2. CHO cell lines were maintained in medium containing 1:1 DMEM-Ham's F12, 5% (v/v) FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Fibroblasts cell lines were grown in DMEM with 10% (v/v) FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Immunocytochemistry

On day 0, CHO cell lines were plated at 1.4×10^4 cells/ml on gelatin-coated 12 mm glass coverslips in 24-well dishes in lipoprotein-containing medium. On day 1, the cells were refed medium containing LPDS. On day 2, the medium was replaced with LPDS medium supplemented with 50 μg/ml LDL in the presence or absence of 1 μM GW707 or 0.5 μM U18666A. Conditions for fixation and antibody and filipin staining have been described previously (25). Cells were examined by fluorescence microscopy on a Zeiss Axiovert epifluorescence microscope. The following filter sets (Chroma) were used: for filipin, excitation filter 360/40 nm, beamsplitter 400 nm, emission filter 460/50 nm; for LysoSensor, excitation filter 470/40 nm, beamsplitter 500 nm, emission filter 535/40 nm; and for Cy3, excitation filter 535/50 nm, beamsplitter 565 nm, emission filter 590 nm.

Cholesterol esterification assays

Cholesterol esterification assays were performed as previously described (7). After lipid extraction, a chromatography recovery standard was added (30 μg of cholesteryl oleate and 0.0005 μCi of [^{14}C]cholesteryl oleate) and samples were dried under nitrogen. The lipids were separated by TLC (PE SIL G plates from Whatman) using heptane-ethyl ether-acetic acid (90:30:1) and visualized with iodine. [^{3}H]Cholesteryl oleate was quantified by liquid scintillation counting. After lipid extraction, monolayers were incubated with 0.1 N NaOH and protein determination was performed using the MicroBCA assay (Pierce).

Luciferase reporter assays

For quantification of SREBP-dependent gene expression, CHO and M12 cells (1×10^6 cells) were electroporated using nucleofection (Amaxa) with 2 μg of LDLp-588luc and 0.5 μg of pRL-TK and then plated at 1×10^5 cells/35 mm well. For experiments shown in Fig. 5A and D, cells were fed LPDS medium on day 2, and on day 3 they were refed LPDS medium in the presence or absence of GW707 (1 μM) and U18666A (0.5 μM). After 24 h, cells were incubated with LDL (50 μg/ml) in the presence or absence of the compounds for an additional 6 h. For the experiment shown in Fig. 5B, cells were fed LPDS medium on day 2, and on day 3 they were refed LPDS medium in the presence of LDL (50 μg/ml) (lipoprotein-fed) or 1 μM GW707 or 20 μM compactin and 50 μM mevalonate (lipoprotein-starved). For the experiment shown in Fig. 5C, cells were fed LPDS medium on day 2, and on day 3 they were refed LPDS medium supplemented with LDL (0–80 μg/ml) in the presence or absence of GW707 (1 μM) and U18666A (0.5 μM). For all experiments, cell lysates were harvested on day 4 and luciferase and *Renilla* activity (Promega) were determined. Normalization of luciferase activity to *Renilla* activity controlled for transfection efficiency.

Gas chromatography-mass spectrometry determinations

For oxysterol measurements, fibroblasts were grown in DMEM/5% LPDS medium for 48 h and then refed DMEM/5% LPDS medium containing 50 μg/ml LDL for 24 h. Oxysterols were extracted from the cells and media as described (26, 27). Because 24-HC was not detected in the fibroblasts, we used 50 pmol of 24-HC as an internal standard during oxysterol isolation. Oxysterols were derivatized to trimethylsilyl ethers by treatment with Sigma Sil-A for 1 h at 60°C. Derivatized samples were analyzed on a Varian 3400 gas chromatograph interfaced to a Finnigan SSQ 7000 mass spectrometer. The gas chromatography column used for the study was a DB-1 (12.5 m, 0.2 mm inner diameter, 0.33 μm film coating; P.J. Cobert, St. Louis, MO). A gradient was run as follows. The initial temperature of 180°C was held for 1 min and increased to 250°C at 20°C/min. The temperature was increased from 250°C to 300°C at 5°C/min and held for 10 min. The mass spectrometer was operated in the electron ionization mode, and the source temperature, electron energy, and emission current were 200°C, 100 eV, and 300 μA, respectively. The injector and transfer line temperatures were 250°C. The presence of 27-HC and 25-HC was monitored with ions at *m/z* 456 at 16.3 min and *m/z* 131 at 15.6 min, respectively. Quantitative gas chromatography-mass spectrometry determinations for 25-HC and 27-HC were calculated from triplicate injections.

Statistics

All results are expressed as means ± SEM. The statistical significance of differences in mean values was determined by one-way ANOVA. Data shown are representative of at least two similar experiments.

RESULTS

Treatment with the steroidal analog GW707 disrupts trafficking of LDL cholesterol

To explore the cellular mechanism of action of the GW707 compound, we examined the effect of treatment with GW707 on intracellular cholesterol trafficking. Wild-type CHO, M12 (NPC1-null), and human NPC1-expressing CHO (CHO/NPC1) cells treated with 1 μM GW707 or 0.5 μM U18666A were costained with filipin, a specific fluorescent marker of unesterified cholesterol, and antisera to human NPC1 and LAMP-2, a late endosomal/lysosomal marker. Using immunofluorescence microscopy, we found that CHO/NPC1 cells incubated with GW707 accumulate free cholesterol in aberrant LAMP-2-positive, NPC1-positive organelles, similar to the cholesterol-laden compartment present in NPC1-null cells and in cells incubated with U18666A (**Fig. 2**). To further demonstrate that GW707 disrupts NPC1-mediated sterol trafficking, we examined the effect of the compound on the rate of LDL-stimulated cholesterol esterification. Cells treated with GW707 show dose-dependent inhibition of LDL cholesterol esterification, similar to that observed in U18666A-treated cells (**Fig. 3**). Moreover, at the highest concentrations examined, both compounds result in severe impairment of LDL cholesterol esterification, characteristic of NPC1-null cells. Although there is a steeper dose response in the U18666A-treated cells, these findings suggest that GW707 impairs mobilization of lysosomal cholesterol in an amphiphile-like manner.

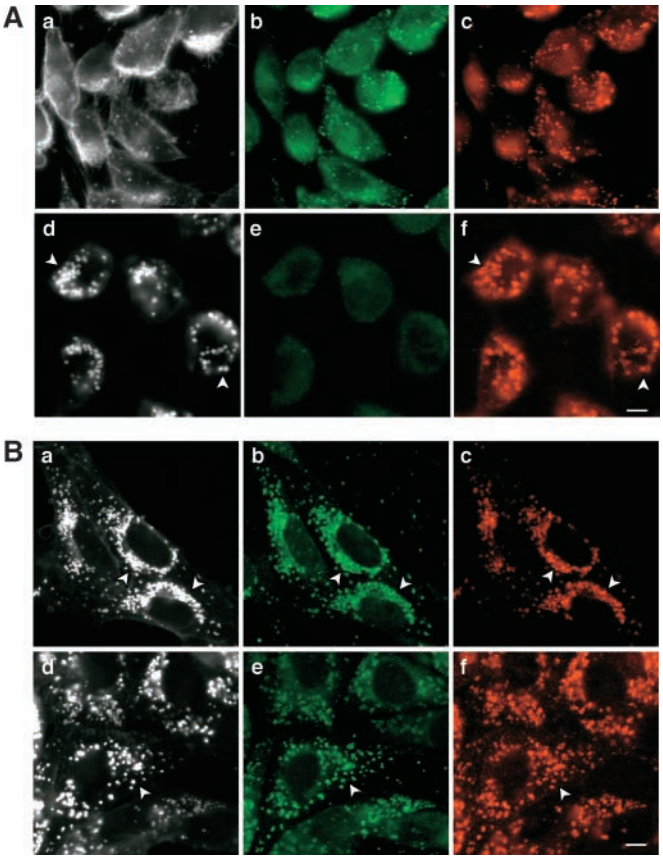

Fig. 2. GW707 induces free cholesterol accumulation in a lysosomal compartment. A: CHO/NPC1 cells (a–c) and NPC1-null cells (d–f) were cultured in lipoprotein-deficient serum (LPDS) medium, pulsed with LDL (50 μg/ml), and costained for cholesterol (a and d), NPC1 (b and e), and LAMP-2 (c and f). Cells were examined by fluorescence microscopy. Note the absence of NPC1 staining in the NPC1-null cells (e). Arrowheads identify LAMP-2 staining at the limiting membrane of cholesterol-laden lysosomes in NPC1-null cells. For each set of panels (a–c and d–f), the images are from a single field. Bar = 10 μm. B: CHO/NPC1 cells were cultured in LPDS medium containing 1 μM GW707 (a–c) or 0.5 μM U18666A (d–f), pulsed with LDL (50 μg/ml), and costained for cholesterol (a and d), NPC1 (b and e), and LAMP-2 (c and f). Cells were examined by fluorescence microscopy. Arrowheads identify colocalization of NPC1 and LAMP-2 in cholesterol-laden lysosomes. For each set of panels (a–c and d–f), the images are from a single field. Bar = 10 μm.

Lysosomal accumulation of cholesterol in GW707-treated cells is SCAP-independent

Because GW707 has been proposed to modulate cholesterol homeostasis by acting as a ligand for SCAP, it is possible that upregulation of cholesterol synthesis and uptake occurs in GW707-treated cells through SCAP/SREBP and is responsible for lysosomal cholesterol accumulation in compound-treated cells. To determine whether SCAP is required for the cholesterol accumulation phenotype, we examined the effect of GW707 on sterol trafficking in 25-RA cells, which harbor the activating D443N SCAP mutation, and in SCAP-null SRD-13A cells (21, 22). 25-RA and SRD-13A cells were fed LDL in the presence or absence of GW707 and then stained for filipin (**Fig. 4**). Immunofluorescence staining revealed GW707-dependent accumulation of unesterified lysosomal cholesterol in both cell lines, identical to that observed in wild-type cells. Free cholesterol accumulation in lysosomes was not observed in the absence of LDL (data not shown). These findings demonstrate that GW707 disrupts sterol trafficking in CHO cells in a SCAP-independent manner.

GW707 prevents LDL cholesterol suppression of SREBP-dependent gene expression

In NPC mutants, the sterol-trafficking defect results in failure to suppress SREBP-dependent gene expression and to promote LXR-mediated responses (20). We reasoned that in GW707-treated cells, which exhibit an NPC-like phenotype, disruption of sterol trafficking similarly prevents appropriate regulation of sterol-regulated gene expression. To examine the effect of treatment with GW707 on SREBP-dependent gene transcription, we used a sterol regulatory element (SRE)-containing reporter construct as an indicator of the status of SREBP maturation (3). Initially, we performed these studies in lipoprotein-fed cells to replicate the assay conditions of the screen through which the LDLr upregulators were identified (**Fig. 5A**) (3). In LDL-fed CHO cells, we found that incubation with 1 μM GW707 and 0.5 μM U18666A leads to a 1.6- and 2.2-fold increase in SRE reporter activity, respectively, comparable to the 1.7-fold activation observed in NPC1-null CHO cells.

In light of the sterol-trafficking defect in the compound-treated and NPC1 mutant cells, we hypothesized that the increase in SRE reporter activity in the LDL-fed cells was not attributable to additional activation of SRE-dependent gene transcription but rather to a failure to appropriately suppress SRE-dependent activity in response to LDL cholesterol. To test this hypothesis, we examined the effect of GW707 on SRE reporter activity under conditions of lipoprotein starvation (Fig. 5B). CHO cells transfected with the SRE reporter were incubated in lipoprotein-deficient medium in the presence or absence of GW707, and SRE reporter activity was determined. In the absence of LDL suppression, no increase in SRE activity was observed in the GW707-treated cells compared with untreated CHO cells. Note that additional activation of SRE-dependent activity was achieved by complete sterol starvation (cells incubated in lipoprotein-deficient medium plus compactin and mevalonate). To demonstrate that GW707 prevents the suppression of sterol-regulated gene expression by LDL cholesterol, we next measured SRE reporter activity in CHO and NPC1-null cells that were incubated in lipoprotein-deficient medium in the presence or absence of GW707 and U18666A, followed by LDL feeding (Fig. 5C). Compared with wild-type CHO cells, GW707- and U18666A-treated cells only partially suppress SRE-dependent activity in response to LDL cholesterol, and NPC1-null cells are resistant to suppression of SRE-dependent activity by LDL cholesterol over the range of concentrations examined. Treatment of NPC1-null cells with GW707 did not result in a further increase

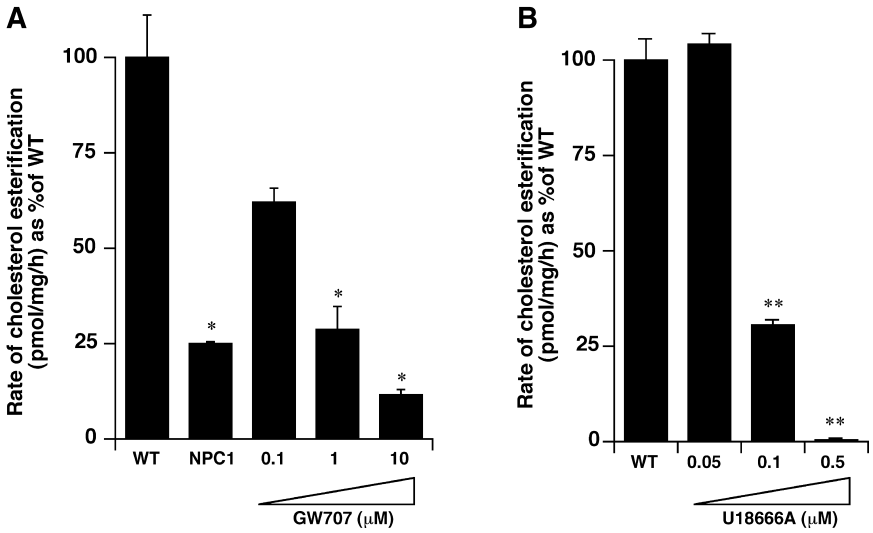

Fig. 3. Inhibition of LDL cholesterol esterification by GW707 and U18666A. CHO and NPC1-null cells were plated in triplicate (2.5×10^4 cells/well) and then grown in LPDS medium for 48 h. CHO cells were fed medium supplemented with LDL in the presence or absence of GW707 (A) and U18666A (B) for 16 h and pulsed for 2 h with [^{3}H]oleate, and the rate of incorporation of [^{3}H]oleate into cholesteryl-[^{3}H]oleate was determined. Esterification rates (picomoles per minute per milligram) are normalized to untreated CHO cells. Values are means ± SEM and are representative of two independent experiments. * $P < 0.005$ versus wild-type (WT) CHO cells; ** $P < 0.001$ versus WT CHO cells.

in SRE reporter activity (Fig. 5D; P = NS for treated versus untreated NPC1-null cells). These findings imply that induction of an NPC-like sterol-trafficking defect in GW707-treated cells is sufficient to explain the apparent activation of SREBP-dependent gene expression.

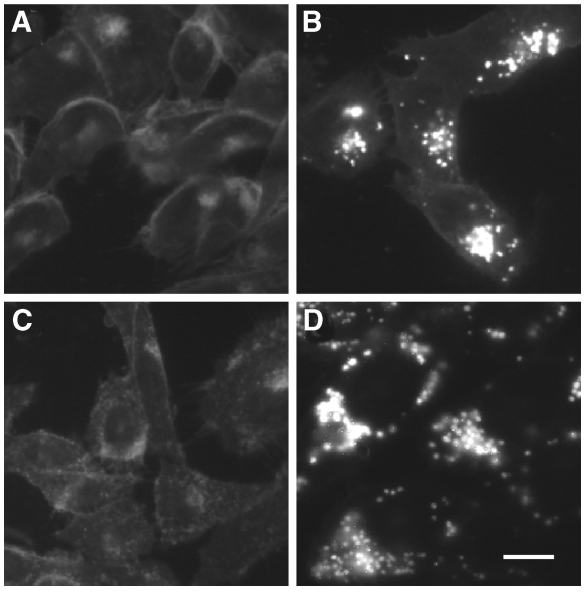

Fig. 4. Free cholesterol accumulation in GW707-treated cells is sterol regulatory element binding protein cleavage-activating protein (SCAP)-independent. 25-RA cells (D443N SCAP mutation) (A and B) and SRD-13A (SCAP-null) cells (C and D) were cultured in LPDS medium plus LDL (50 μg/ml) in the absence (A and C) or presence (B and D) of 1 μM GW707 and stained for cholesterol. Bar = 10 μm.

GW707 inhibits the production of LDL cholesterol-derived oxysterols

Recently, we have shown that cells with NPC1 or NPC2 loss of function are deficient in the generation of oxysterols in response to LDL cholesterol (20). To determine whether treatment with GW707 similarly interferes with the delivery of free cholesterol substrate to sites of cellular oxysterol synthesis, we measured production of 25-HC and 27-HC in wild-type human fibroblasts in the presence and absence of GW707 and U18666A and in NPC1-null and NPC2-null fibroblasts. The rate of 25-HC secretion into the media was decreased by 54% and 70% in the NPC1 and NPC2 mutants, respectively (**Fig. 6**). A similar reduction was observed in U18666A-treated cells (40% decrease), but not in cells exposed to GW707 (5% decrease; P = NS). In contrast, the rate of 27-HC secretion was markedly reduced in both GW707- and U18666A-treated cells (87% and 96% decrease, respectively), comparable to the decrease observed in the NPC1 and NPC2 mutants. Therefore, although both GW707 and U18666A disrupt the delivery of free cholesterol to the mitochondrial sterol 27-hydroxylase, only U18666A appears to interfere with cholesterol delivery to the endoplasmic reticulum (ER)/Golgi-localized cholesterol 25-hydroxylase (28).

DISCUSSION

In the present study, we examined the mechanism through which the steroid analog GW707 exerts its lipid-lowering effects. We show in CHO cells that treatment with GW707 leads to the accumulation of free cholesterol

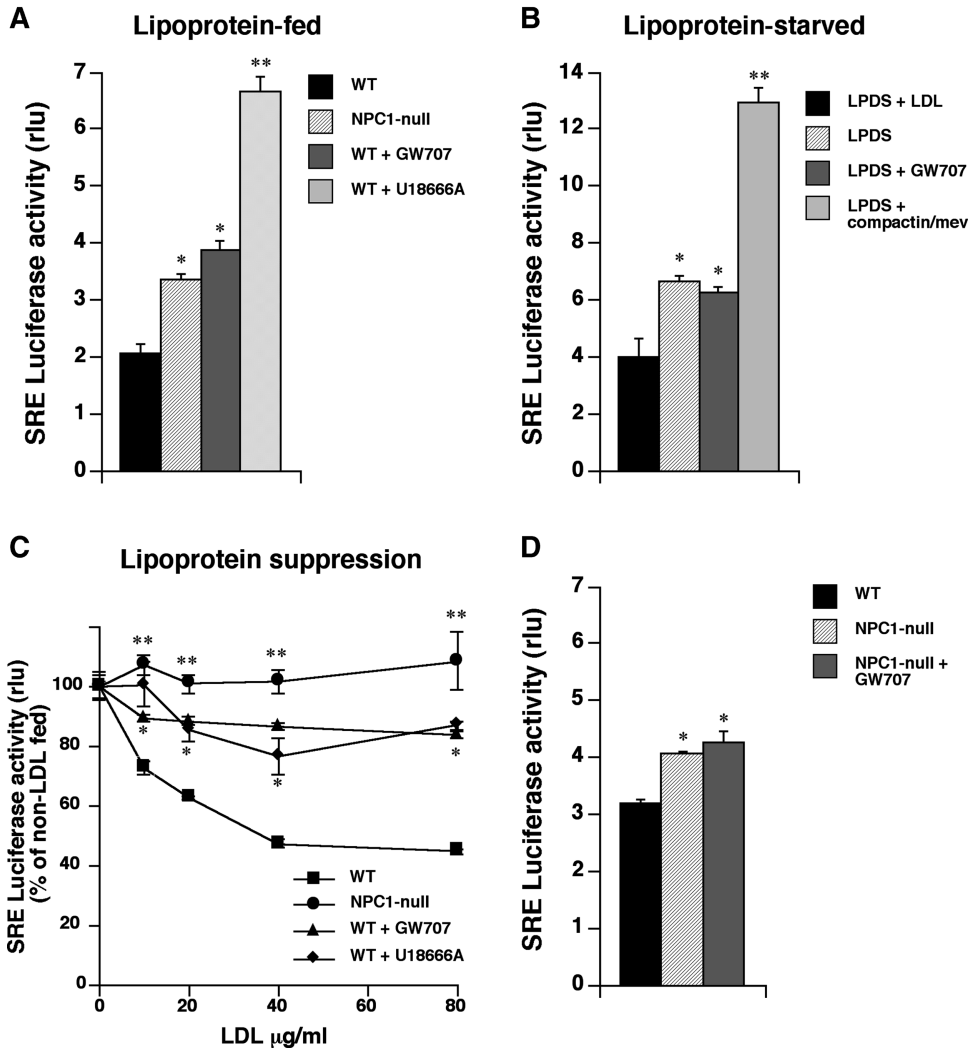

Fig. 5. GW707-treated cells fail to suppress sterol regulatory element (SRE)-dependent gene expression. Cells were cotransfected with a luciferase reporter driven by the human LDL receptor promoter and with a thymidine kinase (TK)-*Renilla* transfection control. A: Lipoprotein-fed conditions. CHO (WT) and NPC1-null cells were grown in LPDS medium for 24 h in the presence or absence of 1 μM GW707 or 0.5 μM U18666A and then incubated with LDL for an additional 6 h in the presence or absence of the compounds. Lysates were harvested, and luciferase and *Renilla* activity was determined. Data are presented as relative light units. Luciferase activity is normalized to *Renilla* activity. Values are means ± SEM and are representative of two independent experiments. * $P < 0.005$ versus untreated WT CHO cells; ** $P < 0.001$ versus untreated WT CHO cells. B: Lipoprotein-starved conditions. CHO cells were cultured in LPDS medium in the absence (LPDS) or presence of LDL (LPDS + LDL), 1 μM GW707 (LPDS + GW707), or 20 μM compactin and 50 μM mevalonate (LPDS + compactin/mev). Luciferase activity was determined as described above. Values are means ± SEM and are representative of two independent experiments. * $P < 0.025$ versus LDL-fed cells; ** $P < 0.002$ versus LDL-fed cells. C: Lipoprotein-suppression conditions. Cells were grown in LPDS medium supplemented with LDL (0–80 μg/ml) in the presence or absence of GW707 (1 μM) and U18666A (0.5 μM). Lysates were harvested and luciferase activity determined as described above. Values are means ± SEM and are representative of four independent experiments. * $P < 0.02$ versus WT CHO cells; ** $P < 0.002$ versus WT CHO cells. D: CHO and NPC1-null cells were grown in LPDS medium for 24 h in the presence or absence of 1 μM GW707 and then incubated with LDL for an additional 6 h in the presence or absence of the compounds. Luciferase activity was determined as described above. Values are means ± SEM and are representative of two independent experiments. * $P < 0.005$ versus WT cells.

in lysosomes and impaired bulk cholesterol esterification, similar to the findings in cells with mutations in the NPC disease genes. Moreover, induction of the NPC-like phenotype by GW707 is SCAP-independent. In cells treated with GW707, the apparent increase in sterol-regulated activity does not result from the activation of the SCAP/SREBP pathway but from the failure of LDL cholesterol to appropriately suppress SREBP-dependent gene expression. In addition, generation of LDL cholesterol-derived 27-HC is markedly impaired in GW707-treated cells. Taken

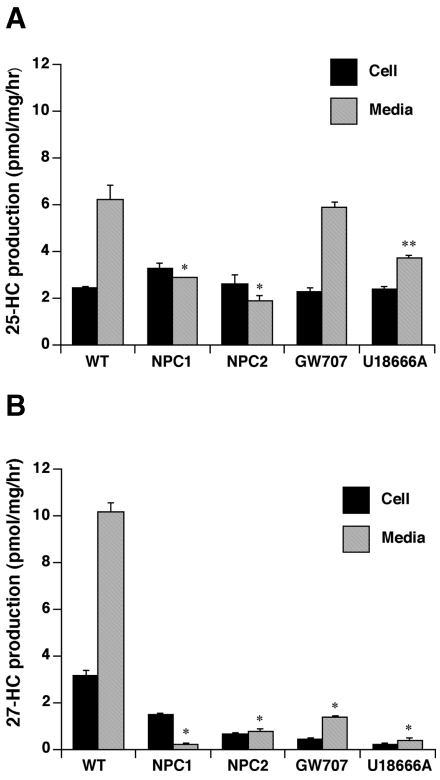

Fig. 6. Determination of oxysterol production in fibroblasts treated with GW707 and U18666A in response to LDL cholesterol. WT, NPC1-null, and NPC2-null fibroblasts were lipoprotein starved for 24 h and refed LPDS medium supplemented with LDL (50 μg/ml) in the presence or absence of 1 μM GW707 or 0.5 μM U18666A. Quantitative gas chromatography-mass spectrometry determinations for 25-hydroxycholesterol (25-HC) and 27-hydroxycholesterol (27-HC) were calculated from triplicate injections and represent total oxysterols in cells and secreted into media. A: Rate of 25-HC production in cells and secretion into the media for WT, NPC1-null, NPC2-null, GW707-treated, and U18666A-treated fibroblasts. * $P < 0.01$ versus WT; ** $P < 0.03$ versus WT. B: Rate of 27-HC production in cells and secretion into the media for WT, NPC1-null, NPC2-null, GW707-treated, and U18666A-treated fibroblasts. * $P < 0.0001$ versus WT. All values are means ± SEM and are representative of two independent experiments.

together, our findings indicate that the GW707 compound stimulates sterol-regulated gene expression in an indirect manner through disruption of intracellular cholesterol trafficking.

GW707 was identified through a screen for compounds that activate expression from the human LDLr promoter (3). It has been proposed that these compounds upregulate LDLr activity based on their ability to bind SCAP and to promote SREBP maturation (3). Several lines of evidence, however, suggest that the GW707 compound modulates sterol homeostasis through an alternative mechanism. First, the screen that identified the LDLr upregulators was designed to detect transcriptional activators of the LDLr. Because the assay was performed under sterol-loading conditions, it is likely that the screen would have also identified classes of compounds that interfere with the endocytic processing of LDL cholesterol and block the delivery of cholesterol to the ER, thereby preventing downregulation of SREBP-dependent gene expression. Our studies support such as mechanism of action for GW707. Second, treatment with GW707 leads to the accumulation of free cholesterol in lysosomes, even in the absence of a functional SCAP. Therefore, induction of the sterol-trafficking defect by GW707 is sufficient to explain the upregulation of LDLr activity without the need to consider direct effects of the compound on SCAP. The finding that treatment with GW707 does not further augment sterol-regulated activity in NPC1-null cells supports this conclusion. Third, in GW707-treated cells, the profound deficiency in 27-HC synthesis suggests that this compound may exert its effects by preventing oxysterol-mediated suppression of sterol-regulated expression, similar to the mechanism proposed for NPC mutants (20).

Treatment with LDLr upregulators in vivo results in a marked reduction in the level of VLDL and LDL (3). In the treated animals, this hypolipidemic response is accompanied by an ~3- to 3.5-fold increase in hepatic LDLr and HMG-CoA reductase mRNA levels. Although these observations imply an important role for the LDLr pathway in the clearance of apolipoprotein B-containing lipoproteins in the SCAP ligand-treated animals, the relative contribution of rates of lipoprotein clearance versus production has not been measured directly. In the liver tissue of treated animals, no increase in cholesteryl esters was observed, although the effect of the compound on free cholesterol levels in this setting is not known. Nonetheless, the effect of LDLr upregulators on lipoprotein metabolism in vivo could be attributed to a defect in the trafficking of LDL cholesterol and is consistent with the cellular mechanism of action we have proposed for GW707.

The steroid analog GW707 exhibits properties consistent with class II amphiphiles, a class of agents that includes steroids and hydrophobic amines (29). Although there is structural diversity among the amphiphiles, GW707 and U18666A, a prototypic class II agent, share common features. Both compounds possess an ether linkage at the C-3 position and alkyl side chain attached to the steroid A-ring that contains a tertiary amine. The compounds differ in the treatment of the sterol side chain: GW707 has two heterocyclic spiro rings at the C-17 position, whereas in U18666A the side chain is absent, indicating that the identity of the C-17 substituent is relatively unimportant compared with the steroid A-ring modifications. The observation that GW706, an inactive analog of GW707 that lacks the alkyl side chain and tertiary amine, neither induces cholesterol accumulation nor inhibits cholesterol esterification supports this conclusion (J. Zhang and D. S. Ory, unpublished observations) (3). Functionally, GW707 and U18666A both disrupt late endosomal/lysosomal trafficking of LDL cholesterol, inhibiting the egress of free cholesterol from lysosomes and bulk cholesterol esterification. Furthermore, both agents prevent the suppression by LDL cholesterol of sterol-regulated gene expression, as indicated by the failure to downregulate LDLr and HMG-CoA reductase activity in compound-treated cells (3, 4). These findings correlate with the ability of both agents to profoundly inhibit the production of 27-HC in response to LDL cholesterol.

On the other hand, GW707 differs from U18666A in several respects. In CHO cells, GW707 is approximately 10-fold less potent than U18666A with respect to inhibition of LDL-stimulated cholesterol esterification, whereas in HepG2 cells, GW707 is 2- to 3-fold more potent than U18666A in inhibition of cholesteryl ester synthesis (E. Lopez-Perez and M. Issandou, unpublished data). In addition, U18666A, unlike GW707, impairs cholesterol biosynthesis through the inhibition of 2,3-oxidosqualene cyclase (30). In the present study, we found that GW707 does not significantly affect the synthesis of 25-HC in response to LDL cholesterol, whereas U18666A-treated cells exhibit a 40% reduction in the rate of secretion of 25-HC. There are several possible explanations for the divergent effects of the compounds on 25-HC synthesis. GW707 may selectively inhibit trafficking of LDL cholesterol to the mitochondrial sterol 27-hydroxylase, whereas treatment with U18666A may interfere more broadly with the delivery of free cholesterol to sites of cellular oxysterol synthesis. Alternatively, the compounds may differentially affect the trafficking or presentation of the cholesterol substrate to sterol 27-hydroxylase. Given the ability of the enzyme to catalyze both 25- and 27-hydroxylation (31), it is possible that U18666A, but not GW707, affects the relative rate of 25-hydroxylation. At present, however, little is known of the mechanisms involved in the regulation of oxysterol synthesis. In future studies, GW707 may be used to probe specific limbs of the sterol-trafficking pathway or to explore the mechanism of the sterol 27-hydroxylase.

The molecular mechanism through which the amphiphilic compounds exert their effects is not known. The observation that cells overexpressing the NPC1 protein are resistant to the effects of U18666A implies that NPC1 or a protein in the same pathway may be the molecular target of this class of compounds (8). The finding that LDLr upregulators can bind to the sterol-sensing domain of SCAP raises the possibility that amphiphilic compounds likewise may interact with the sterol-sensing domain of NPC1, which is 28% identical and 54% similar to the homologous region of SCAP. Alternatively, amphiphiles may target the NPC2 protein, which has been shown to bind free cholesterol with high affinity (14, 15). Future studies examining the mechanism of action of these compounds may identify specific inhibitors of NPC1 or NPC2 function and will further our understanding of the role of the NPC proteins in the regulation of sterol trafficking.

The authors are grateful to D. Covey for helpful discussions. D.S.O. is supported by Grants HL-04482 and HL-67773 from the National Institutes of Health, and J.R.C. is supported by Grants DK-6341 and P-41-RR-00954 from the National Institutes of Health.

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