Glucagon receptor antagonism induces increased cholesterol absorption

Hong-Ping Guan,†,§ Xiaodong Yang,*, Ku Lu,*, Sheng-Ping Wang,*, Jose M. Castro-Perez,*, Stephen Previs,*, Michael Wright,† Vinit Shah,*, Kithsiri Herath,*, Dan Xie,*, Daphne Szeto,§ Gail Forrest,§ Jing Chen Xiao,*, Oksana Palyha,*, Li-Ping Sun,*, Paula J. Andryuk,** Samuel S. Engel,§ Yusheng Xiong,*, Songnian Lin,†† David E. Kelley,*, Mark D. Erion,† Harry R. Davis,‡,* and Liangsu Wang*<sup>†</sup>

Departments of Cardiometabolic Disease,† Late Stage In Vitro Pharmacology,† Late Stage In Vivo Pharmacology,§ and Discovery Chemistry,‡† Merck Research Laboratories, Kenilworth, NJ 07033; and Clinical Research Department,** Merck Research Laboratories, Rahway, NJ 07065

Abstract Glucagon and insulin have opposing action in governing glucose homeostasis. In type 2 diabetes mellitus (T2DM), plasma glucagon is characteristically elevated, contributing to increased gluconeogenesis and hyperglycemia. Therefore, glucagon receptor (GCGR) antagonism has been proposed as a pharmacologic approach to treat T2DM. In support of this concept, a potent small-molecule GCGR antagonist (GRA), MK-0893, demonstrated dose-dependent efficacy to reduce hyperglycemia, with an HbA1c reduction of 1.5% at the 80 mg dose for 12 weeks in T2DM. However, GRA treatment was associated with dose-dependent elevation of plasma LDL-cholesterol (LDL-c). The current studies investigated the cause for increased LDL-c. We report findings that link MK-0893 with increased glucagon-like peptide 2 and cholesterol absorption. There was not, however, a GRA-related modulation of cholesterol synthesis. These findings were replicated using structurally diverse GRAs. To examine potential pharmacologic mitigation, coadministration of ezetimibe (a potent inhibitor of cholesterol absorption) in mice abrogated the GRA-associated increase of LDL-c. Although the molecular mechanism is unknown, our results provide a novel finding by which glucagon and, hence, GCGR antagonism govern cholesterol metabolism.—Guan, H.-P., X. Yang, K. Lu, S.-P. Wang, J. M. Castro-Perez, S. Previs, M. Wright, V. Shah, K. Herath, D. Xie, D. Szeto, G. Forrest, J. C. Xiao, O. Palyha, L.-P. Sun, P. J. Andryuk, S. S. Engel, Y. Xiong, S. Lin, D. E. Kelley, M. D. Erion, H. R. Davis, and L. Wang. Glucagon receptor antagonism induces increased cholesterol absorption. J. Lipid Res. 2015. 56: 2183–2195.

Supplementary key words diabetes • glucagon receptor antagonist • cholesterol/absorption • hypercholesterolemia • glucagon-like peptide 2 • bile acids

It is through mostly opposing actions that the pancreatic islet hormones, insulin and glucagon, interact in the governance of hepatic glucose production and its uptake. In type 2 diabetes mellitus (T2DM), as well as in type 1 diabetes mellitus, fasting plasma glucagon is generally elevated, inappropriate to prevailing hyperglycemia, and there is less suppression during prandial metabolism (1). This imbalance in secretion of islet hormones is considered to be a key aspect of the pathophysiology causing hyperglycemia (1, 2). Glucagon receptor (GCGR) antagonism has accordingly drawn considerable interest as a novel pharmacological approach for treating T2DM. Several GCGR antagonists (GRAs) have advanced into human clinical trials in patients with T2DM. MK-0893 (3), MK-3577 (4), LY2409021 (5, 6), and an anti-sense oligo targeting the GCGR (ISIS-GCGRx) (7) have each demonstrated efficacy in lowering fasting and postprandial hyperglycemia, leading to substantial reductions of HbA1c, thereby providing clinical proof of concept for the efficacy of GRAs.

In a 12 week placebo-controlled dose-ranging clinical study in T2DM using the GRA, MK-0893, dose-response improvement of hyperglycemia was observed, with a reduction of HbA1c of 1.5% at 80 mg per day, the top dose

Abbreviations: Cyp7a1, Cytochrome P450 family 7 subfamily A polypeptide 1; Cyp7b1, Cytochrome P450 family 7 subfamily B polypeptide 1; Cyp8b1, Cytochrome P450 family 8 subfamily B polypeptide 1; Cyp27a1, Cytochrome P450 family 27 subfamily A polypeptide 1; Cyp39a1, Cytochrome P450 family 39 subfamily A polypeptide 1; Cyp46a1, Cytochrome P450 family 46 subfamily A polypeptide 1; Cyp51, Cytochrome P450 family 51; GCGR, glucagon receptor; GLP, glucagon-like peptide; GRA, glucagon receptor antagonist; 7-HCO, 7α-hydroxy-4-cholesten-3-one; HDL-c, HDL cholesterol; hGCGR, humanized glucagon receptor; HMGCR, HMG-CoA reductase; LDL-c, LDL cholesterol; LDLR, LDL receptor; PCSK9, propionate convertase subtilisin/kexin type 9; QD, once daily; Srebpr, sterol regulatory element-binding protein; T2DM, type 2 diabetes mellitus.

<sup>†</sup>To whom correspondence should be addressed.
e-mail: Hong-Ping.Guan@merck.com

<sup>‡</sup>Present address of H. R. Davis: CVPath Institute, Inc., 19 Firstfield Road, Gaithersburg, MD 20878.

The online version of this article (available at http://www.jlr.org) contains a supplement.
examined (supplementary Fig. 1) (3). This efficacy is substantial and, arguably, as or more effective than contemporary standard-of-care oral agents for treatment of T2DM. Yet, in association with the dose-responsive improvements in hyperglycemia, a dose-dependent increase in plasma LDL-cholesterol (LDL-c) was observed. At the 80 mg dose, plasma LDL-c increased by 16.7% relative to baseline, significantly greater than under placebo or metformin treatment arms (−3.1 and 2.2% changes, respectively) (supplementary Fig. 1) (3). LDL-c and T2DM are recognized to adversely influence risk for cardiovascular disease, and increased LDL-c in the setting of T2DM is a cause for concern, as this could potentiate the risk for cardiovascular disease (8–10).

The current studies were undertaken using a preclinical rodent model and cholesterol isotopic flux determinations, as well as further exploration of archived plasma samples from the clinical trial with MK-0893, to elucidate the principal mechanism underlying increased plasma LDL-c. A fundamental related question is whether the findings are unique to a specific GRA compound or, instead, represent a mechanism-based response. To address this, several structurally distinct GRAs were investigated for effects on cholesterol homeostasis. The findings yield novel insights into glucagon physiology, as well as GRA pharmacology, and indicate a substantial effect in the regulation of cholesterol absorption.

MATERIALS AND METHODS

Animal studies

All mice used in the studies were purchased from Taconic ( Germantown, NY) at 10–12 weeks of age. Animals were maintained in a 12 h/12 h light-dark cycle with free access to food and water in an environment with temperature maintained at 22°C. Four mice were housed in a regular cage. Male humanized GCGR (hGCGR) mice were generated on a B6.129S6 background and had been backcrossed to C57BL/6 for more than 13 generations. All mice used in the studies were purchased from Taconic (presently Life Technologies, H9262) as this could potentiate the risk for cardiovascular disease, and comparisons were made between the compound treatment and vehicle groups. Animals were maintained on a rodent chow diet 7012 (5% dietary fat; 3.75 kcal/g) (Teklad, Madison, WI) for 2 weeks before receiving compound treatments and vehicle. Groups were performed under ad libitum feeding, as previously described (12). Briefly, at 1 h post compound administration via po, glucagon dissolved in PBS was injected at 15 μg/kg ip followed by glucose measurements using a glucometer (Life Scan) via tail bleeding at 0, 12, 24, and 48 min according to manufacturer’s instructions (WakoUSA). The plasma or serum level of proprotein convertase subtilisin/kexin type 9 (PCSK9) was assayed by extracting lipids using the Folch method (16), whereby the plasma or serum was separated by centrifugation at 8,500 rpm at 4°C and stored at −80°C until assayed. Human serum was collected from CellzDirect (presently Life Technologies, Hu8080). One vial of frozen primary hepatocytes (approximately five million cells in total) was quickly thawed to 37°C in a water bath and washed in cryopreserved hepatocyte recovery medium (Life Technologies, CM7000) and resuspended in buffer containing HBSS (Life Technologies, 14025), 0.1% BSA (Sigma, A9205), and 1.2 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, I-5879). To assess antagonist activity, 4,000 cells per well were preincubated with compounds or 0.1% DMSO for 30 min and stimulated with glucagon (5 nM) (Sigma, G2044) for an additional 30 min at room temperature. The assay was terminated with the addition of Cisbio Dynamic 2 (62AM4PEC) detection reagents, as per the manufacturer’s instructions (Cisbio). cAMP was detected by a decrease in time-resolved fluorescence energy transfer using an EnVision plate reader (PerkinElmer). The IC50 values were calculated using nonlinear regression curve fit analysis in Prism (GraphPad).

Measurement of plasma or serum GLP-1 and GLP-2

Whole blood of mice was collected in EDTA-coated tubes and plasma was separated by centrifugation at 8,500 rpm at 4°C and stored at −80°C until assayed. Human serum was collected following a standard blood collection procedure after overnight fasting. Plasma or serum levels of GLP-1 and GLP-2 were measured using a total GLP-1 assay kit (Meso Scale Discovery) and mouse/human GLP-2 kit (Alpco).

Analysis of plasma lipid, apolipoprotein, PCSK9, and fecal cholesterol

A commercial enzymatic colorimetric kit was used for the determination of plasma total cholesterol (Wako cholesterol E kit) according to manufacturer’s instructions (WakoUSA). The plasma level of proprotein convertase subtilisin/kexin type 9 (PCSK9) was determined by PCSK9 dissociation-enhanced lanthanide fluorescence immunoassay, as described elsewhere (14). The plasma or serum lipoprotein profile was assayed by fast-protein LC, as described previously (15). Fecal cholesterol was measured by extracting lipids using the Folch method (16), whereby fecal samples were homogenized with 5 ml of chloroform:methanol (2:1, v/v). The homogenate was then filtered and washed with 2 ml of 0.9% saline, followed by centrifugation and drying of the lower phase under nitrogen gas. The extract was reconstituted with 10% Triton X-100 in isopropanol and analyzed using a commercial cholesterol kit (WakoUSA).
The ²H-labeling of body water was determined using headspace analyses following exchange with acetone, as described by Shah et al. (17). Briefly, 20 µl of sample (or standard) was reacted with 2 µl of 10 N NaOH and 4 µl of a 5% (v/v) solution of acetone in acetonitrile for 4 h at room temperature. The instrument was programmed to inject 5 µl of headspace gas from the GC via a splitless sample. Samples were analyzed using a 2.0 min isothermal run (Agilent 7973 mass spectrometer coupled to a 6890 GC oven fitted with an Agilent DB-5MS column (30 m × 250 µm × 0.15 µm)); the oven was set at 170°C and helium carrier flow was set at 1.0 ml/min⁻¹, acetone elutes at ~1.4 min; the mass spectrometer was set to perform selected ion monitoring of m/z 58 and 59 (10 ms dwell time per ion) in the electron impact ionization mode.

The isotopic labeling of total cholesterol was determined using GC-MS (18). Lipids were saponified by heating plasma (50 µl) with 1 N KOH in 80% methanol (200 µl) at 65°C for 1 h. Samples were acidified with 25 µl 6 N HCl and then extracted in 125 µl chloroform followed by vigorous vortexing for 20 s. The samples were centrifuged at 3,000 rpm for 5 min and 100 µl of chloroform (lower layer) was collected and evaporated to dryness under N2. Samples were derivatized by reacting with 100 µl of pyridine:acetic anhydride (1:2, v:v) at 65°C for 1 h. Excess reagent was evaporated to dryness under N2 and the acetylated derivative was reconstituted in 50 µl ethyl acetate for analysis by GC-MS. All analyses were performed using an Agilent 5973 mass spectrometer coupled to a 6890 GC oven fitted with an Agilent DB-5MS column (30 m × 250 µm × 0.15 µm). The instrument was programmed to inject 1 µl of sample using a 10:1 split (helium carrier flow was set at 1.0 ml/min⁻¹). The oven temperature was started at 150°C, raised at 20°C/min to 310°C, and held for 6 min; cholesterol elutes at ~9.5 min. The mass spectrometer was set to perform selected ion monitoring of m/z 368 and 369 (10 ms dwell time per ion) in the electron impact ionization mode.

Calculations and statistical analysis

To quantify the contribution of cholesterol synthesis to blood cholesterol level, the data was fit (using a precursor/product labeling ratio) to the general equation (18): newly made cholesterol = [product labeling / (precursor labeling × n)] × concentration, where n is the number of exchangeable hydrogens (assumed to equal 26 for cholesterol) (2). The change in the ratio of m/z 369:368 (i.e., M+1/M0) was used to model the product labeling, whereas the precursor labeling was assumed to equal plasma water. The concentration of total circulating cholesterol was determined via enzymatic assay (19, 20). The plasma level and flux of ApoB were quantified by the LC-MS/MS method, as described previously (21).

Analysis of campesterol and sitosterol in plasma or serum samples of mice and humans. Five microliters of plasma or serum were mixed with 25 µl of internal standard mix (1 µg/ml of D₃-campesterol and D₃-sitosterol prepared in ethanol) and 100 µl of 1 N KOH in glass inserts placed on a deep 96-well polypropylene plate. The mixture was sealed and heated at 80°C with shaking at 600 rpm for 1 h on an R-shaker (Eppendorf). Samples were evaporated to dryness under nitrogen. Derivatization reagent [150 µl (1,000 mg of 2-methyl 6-nitro benzoic anhydride, 300 mg of 4-dimethyl pyridine, and 800 mg of picolinic acid dissolved in 2 ml of triethylamine and 12 ml of pyridine)] was added to each tube and the plate was incubated at 80°C for 1 h. After incubation, 500 µl of hexane was added to each tube, vortexed, and centrifuged at 4,000 rpm at room temperature for 10 min. Supernatant (400 µl) was transferred to a new glass microtube, evaporated to dryness under a constant flow of nitrogen at 45°C, and reconstituted in 80 µl of loading solution (80% acetonitrile, 20% water, and 0.1% formic acid). Samples were then loaded for LC-MS analysis. Contents of campesterol and sitosterol were normalized to the internal controls in each sample. For each assay, five pooled plasma samples from multiple subjects were used as quality controls and each quality control sample was injected in triplicate for LC-MS assay. Quality controls with a variation of ±15% coefficient were deemed as acceptable.

Bile acid and intermediate analysis

Serum (150 µl) was transferred into a deep 2 ml 96-well plate followed by the addition of 585 µl ice-cold acetonitrile containing 0.1% formic acid solution and 5 µl 60 ng/ml internal standard mixture made of d6-7α,12α-dihydroxy-4-cholesten-3-one and d7-7α-hydroxy-4-cholesten-3-one. The plate was sealed and vortexed for 1 min followed by centrifugation at 4,000 rpm for 20 min at room temperature. After centrifugation, 600 µl of supernatant was passed (under positive pressure) through a protein precipitation plate, which retained phospholipids but eluted the bile acid intermediates (Ostro plate; Waters Corp., Milford, MA). The eluent was collected and evaporated under a constant flow of N2 at 45°C. The samples were then reconstituted in 100 µl of 80% acetonitrile and 0.1% formic acid/20% water. The resultant extract (10 µl) was injected onto an LC-MS/MS system operated in positive ion mode electrospray (UPLC/TQS mass spectrometer; Waters Corp.). Isotopic dilution quantitation was conducted to obtain concentrations of 7α,12α dihydroxy-4-cholesten-3-one and 7α-hydroxy-4-cholesten-3-one.

Western blotting

One piece of liver (~100 mg) was homogenized in 500 µl of RIPA buffer by using FastPrep™24 (MP Biomedicals). After incubation on ice for 30 min, homogenate was centrifuged at 14,000 rpm at 4°C for 30 min. The protein concentration of the supernatant was determined by BCA protein assay kit (Pierce) and the final concentration was calibrated to 1 mg/ml with RIPA buffer. After mixing with 2× loading buffer and heating at 70°C for 5 min, samples were loaded at 20 µg per well to a 4–10% SDS-PAGE gel for electrophoresis. After transferring the protein to polyvinylidene difluoride membrane, LDL receptor (LDLR) was blocked by using a rabbit monoclonal antibody (Abcam, ab528188). Loading control was blocked by using β-actin polyclonal antibody (Cell Signaling Technology).

Real-time quantitative PCR analysis and gene profiling

Liver samples isolated from mice treated with vehicle and GRA compound(s) were kept in RNAlater solution (Qiagen) until processing. Tissues were homogenized and total RNA was isolated by using an RNA Easy kit and QIAcube instrument (Qiagen). Total RNA (2 µg) from each sample was reverse transcribed with a cDNA kit (Life Technologies), and mRNA levels for the genes of interest were measured by RT-PCR with TaqMan Universal Master Mix reagents and TaqMan primer/probe sets (Life Technologies) or SYBR Green Master Mix reagents and a custom-designed PCR array developed in collaboration with SABiosciences-Qiagen (22). The relative amounts of specific target amplicons for each gene were estimated by a cycle threshold (CT) value and were normalized to the copy number of housekeeping genes, with all genes in a vehicle group arbitrarily set at one (22). The P values were determined by two-tailed equal variance Student’s t-test, comparing the 2⁻¹⁰⁰ values of the vehicle and GRA-treated groups.

Data analysis and statistics

All data are presented as mean ± SEM. For rodent results, statistical analysis was performed by using one-way ANOVA followed by...
an unpaired two-tailed Student’s t-test to compare mean values between treatment groups and the control group. For human results, due to an uneven number of human samples in different groups, the percent change of the measurements (parameters at 12 weeks vs. day −1) was calculated for each individual and then averaged. Statistical analysis was performed by using the Mann-Whitney test.

Declaration

For rodent studies, all testing protocols were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committees in Rahway and Kenilworth, NJ. The Guide for the Care and Use of Laboratory Animals was followed in the conduct of the animal studies. Veterinary care was given to animals requiring medical attention. Finally, the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), published by NC3Rs, were followed for reporting the in vivo experiments in animal research.

Clinical trial protocols of MK-0893 were reviewed and approved by an independent institutional review board or ethical review committee before being initiated. For each site, the institutional review board/ethical review committee and Merck’s consent form review department (US studies) or local medical director (non-US studies) approved the patient informed consent form. Written informed consent was received from participants prior to inclusion in the study. In all cases, Merck clinical studies were consistent with standards established by the Declaration of Helsinki and in compliance with all local and/or national regulations and directives.

RESULTS

Selection and characterization of GRA test compounds

Three GRA compounds [MK-0893 (23), GRA1 (24), and GRA2 (25)] were demonstrated to potently block glucagon binding to the GCGR and were selected for use in the preclinical studies that comprise this investigation. MK-0893 and GRA1 were developed by Merck and GRA2 is under development by Eli Lilly. GRA2 possesses a biaryl benzyl ether core that is distinctly different from the trisubstituted diazole core present in GRA1 and MK-0893 (Fig. 1). To assess relative potencies, cryopreserved human hepatocytes were incubated with MK-0893, GRA1, or GRA2 in the presence of 5 nM glucagon followed by cAMP quantitation in the culture media. Glucagon induced cAMP production dose dependently, with an EC_{50} of 575 pM, which is comparable to physiological levels of glucagon in blood (2). MK-0893, GRA1, or GRA2 alone had no effect on cAMP production. In the presence of glucagon, each compound suppressed cAMP production, with IC_{50}s of 563, 448, and 292 nM, respectively (Fig. 1). Comparing these results with values earlier obtained from CHO cells stably overexpressing human GCGR (hGCGR).CHO) (23, 24), IC_{50}s of the compounds are right-shifted, likely reflecting overexpression of GCGR in engineered cells versus human primary hepatocytes. Nonetheless, these data indicate the three GRA compounds are comparably potent. To further characterize these compounds, the compounds were compared for effects on in vitro binding of glucagon to GCGR, β-arrestin recruitment, cellular level of Ca^{2+}, and cAMP production in hGCGR.CHO cells (supplementary Table 1). No meaningful differences among the three selected GRAs were observed across these parameters, indicating good similarity of pharmacology of antagonizing GCGR-mediated signaling.

Effect of GRA on cholesterol synthesis and clearance in hGCGR mice

The next prerequisite was to identify a relevant preclinical model. For this purpose, the hGCGR mouse model was chosen (11). The selected GRAs have more potent and specific binding for human than mouse GCGR (24). hGCGR mice are lean, healthy, and without abnormal plasma glucose and lipids (11). To test for qualification of hGCGR mice for these investigations, the mice were maintained.

Fig. 1. GRAs suppress glucagon-induced cAMP production in human primary hepatocytes. Human primary hepatocytes were thawed and washed with CHAMPS buffer followed by cAMP production assay. The assay was done in triplicate. The EC_{50} of glucagon on cAMP production is 575 pM. The IC_{50}s of MK-0893, GRA1, and GRA2 are 563, 448, and 292 nM, respectively. All compound treatments were performed in the presence of 5 nM of glucagon. Dose titration of MK-0893 alone is shown in the panel of MK-0893. All treatments were done in duplicate. Dotted curve is a replication of the glucagon dose response curve in the panel of glucagon.
on chow diet and treated with GRA1 at 30 mpk once daily (QD) for 5 days. Glucagon-induced glucose excursion on day 1 after a single dose and ambient glucose on day 5 were measured as indicators of acute and subchronic GCGR blockade. Glucose excursion induced by glucagon after a single dose was suppressed (Fig. 2A), and ambient glucose decreased by 20% after 5 days (Fig. 2B), demonstrating effective target engagement. On chow diet under GRA1 treatment, plasma levels of cholesterol, LDL-c, and HDL-cholesterol (HDL-c) were increased by 19, 33, and 21%, respectively (compared with vehicle) (Fig. 2B, D; supplementary Fig. 3). Plasma TG level was not affected by GRA1 treatment (supplementary Fig. 3). Taken together, it was concluded that hGCGR mice were a suitable preclinical model for investigating the mechanism of action by which GRA induces a rise in plasma cholesterol, LDL-c, and HDL-c.

It had been reported that glucagon inhibits HMG-CoA reductase (HMGCR) and suppresses cholesterol synthesis (27). Therefore, we initially focused upon testing the hypothesis that GRA increases cholesterol synthesis. Isotope tracer methodology was used. hGCGR mice were administered D2O (20 ml/kg body weight ip) to label newly synthesized cholesterol, palmitate, and ApoB, and determine the rate of

Effects of GRA on cholesterol absorption in hGCGR mice

The next set of studies was undertaken to examine a potential effect of GRA1 on cholesterol absorption and its excretion. An in vivo study performed in hGCGR mice on chow diet was adapted from a study design previously published (28). Cholesterol absorption was measured using a stable
isotope method and cholesterol excretion was monitored by measuring fecal cholesterol. Stable isotope-labeled cholesterol, 2,2,3,4,4,6-D_{6}-cholesterol (15 mpk, po) and 3,4-^{13}C_{2}-cholesterol (15 mpk, iv), were administered on day 1 followed by blood collection at 24, 48, and 72 h post injection. Cholesterol absorption was calculated by the ratio of 2,2,3,4,4,6-D_{6}-cholesterol to 3,4-^{13}C_{2}-cholesterol in plasma at different time points. At 24 h prior to euthanasia, mice were dosed with D_{2}O (ip, 20 ml/kg) for measurement of cholesterol and palmitate synthesis (Fig. 3A). After 9 days of treatment, GRA1 increased plasma levels of total cholesterol by 18%, thus, a highly consistent response (Fig. 3B). Cholesterol absorption was found to be increased by 46% (Fig. 3C). A similar increase of cholesterol absorption was observed for MK-0893 and GRA2 (supplementary Fig. 5). It was again observed that there was not a significant change in the rate of cholesterol synthesis (Fig. 3D). Cholesterol excretion was not found to be significantly changed (Fig. 3E).

**GRA1 and GRA2 increase blood cholesterol and cholesterol absorption**

The key aspect of these studies was to address whether the finding of increased cholesterol absorption was unique for a specific GRA compound or instead could be mechanism based. The isotope study described above was repeated using the structurally distinct GRA2. A second aspect was to add measurements of phytosterols (plant sterols), the plasma levels of which are recognized as biomarkers of intestinal cholesterol absorption (30). GRA1 and GRA2 were dosed (30 mpk each) to achieve similar and robust target engagement, as assessed by blockade of a glucagon-induced glucose excursion (Fig. 4A). After 5 days of treatment, GRA1 and GRA2 increased plasma total cholesterol by 20 and 27.3%, respectively (Fig. 4B). Both compounds increased cholesterol absorption, as indicated by treatment-related increases in plasma phytosterols. Campesterol increased 33.1% for GRA1 and 33.3% for GRA2, and plasma sitosterol increased 37.2% for GRA1 and 38.9% for GRA2 (Fig. 4B). Based on D_{2}O labeling, no significant change in the rate of cholesterol synthesis was found for either GRA1 or GRA2. GRA2 slightly increased the plasma level of lathosterol; however, there was not a significant difference in the lathosterol-to-cholesterol ratio on treatment with GRA1 or GRA2 (Fig. 4B), an indirect measurement of cholesterol synthesis (31).

It was reported that plasma levels of GLP-1 and GLP-2 were dramatically increased in GCGR^{−/−} mice (32, 33). In hGCGR mice treated with GRA1 and GRA2, plasma levels of total GLP-1 were increased by 398 and 674%, respectively, and levels of GLP-2 were increased by 71 and 96%, respectively (Fig. 4C), consistent with the pattern found in clinical samples from the MK-0893 clinical trial (Fig. 5C). In a separate study in hGCGR mice, MK-0893 treatment increased the plasma level of total GLP-1 by 168% and of GLP-2 by 37% (supplementary Fig. 7).

As a corollary experiment to determine whether the observed effects on cholesterol homeostasis are compound specific, we sought to exploit that MK-0893 achieves...
Glucagon receptor antagonists induce cholesterol absorption

Glucagon receptor antagonists induce cholesterol absorption 2

MK-0893 increases phytosterols, GLP-1, GLP-2, and bile acids in T2DM

Substantially more robust binding to human GCGR than mouse GCGR by examining the respective treatment effect upon cholesterol in WT versus hGCGR mice. We posited that if the increase in cholesterol absorption was an “off-target” effect, then resultant increases in plasma cholesterol would be similar in WT and hGCGR mice. Doses of 3 and 10 mpk were used for MK-0893 in WT and hGCGR mice, but robust target engagement (glucose response to glucagon challenge) was demonstrable at both doses only in hGCGR mice. MK-0893 had no significant effect on plasma cholesterol or phytosterols in WT mice, but demonstrated a significantly increased plasma level of campesterol at 10 mpk and a trend for dose-dependent increase in plasma sitosterols in hGCGR mice (supplementary Fig. 2). Taken together, these studies bolster the concept that blockade of GCGR induces an increase in cholesterol absorption and plasma cholesterol as a mechanism-based effect.

MK-0893 increases phytosterols, GLP-1, GLP-2, and bile acids in T2DM

Having generated a hypothesis based on pharmacologic interventions in hGCGR mice that GRA induces increased cholesterol absorption and, importantly, established utility of using phytosterols as relevant biomarkers of this process, we were then positioned for further examination of archived plasma samples obtained during the MK-0893 clinical study (ClinicalTrials.gov identifier: NCT00479466). In this trial, placebo, metformin, and MK-0893 at 20, 40, 60, and 80 mg were chosen to treat T2DM (3). Archived samples from the placebo and the MK-0893 60 mg and 80 mg treatment arms were assayed for glucose, total cholesterol, GLP-1, GLP-2, LDL-c, phytosterols, bile acids, and bile acid metabolites (Fig. 5, supplementary Fig. 4). The 60 mg treatment with MK-0893 decreased glucose by 31% (versus a 3.6% rise with placebo) and increased LDL-c by

![Graphs showing glucose, cholesterol, and phytosterol levels](image-url)
have a critical role in facilitating absorption of highly hydrophobic cholesterol. Though an effect of GRA on bile acid metabolism has not previously been reported, the plasma samples were examined for total bile acid concentration, which was found to be significantly increased by MK-0893 (Fig. 5E). In particular, there was an increase of cholic acid and in 7-HCO, a biochemical intermediate in the rate-limiting reaction converting cholesterol to bile acids. The 7,12-diHCO, an intermediate in cholic acid synthesis, showed a trend for increase (Fig. 5E, supplementary Fig. 4E, F). These results augment the finding that GRA induces an increase in cholesterol absorption and suggest that GRA mediates an increase of bile acid synthesis.

4.2% (versus a 4.8% decline with placebo), as shown in Fig. 5A. Serum campesterol and sitosterol were significantly increased with MK-0893 treatment (Fig. 5D).

It is recognized that GRA or genetic knockout of GCGR evokes large compensatory increases in plasma glucagon, together with increased expression for other peptides derived from preproglucagon. It has been reported that GLP-2 enhances nutrient absorption and induces epithelial cell proliferation and regeneration (34–37). In the archived plasma samples, we observed a treatment-related increase of total GLP-1 (6-fold for 60 mg and 12-fold for 80 mg) and a less pronounced increase in plasma GLP-2 (1.4-fold for 60 mg and nearly 3-fold for 80 mg) (Fig. 5B, C). In the aqueous environment of the small intestine, bile acids have a critical role in facilitating absorption of highly hydrophobic cholesterol. Though an effect of GRA on bile acid metabolism has not previously been reported, the plasma samples were examined for total bile acid concentration, which was found to be significantly increased by MK-0893 (Fig. 5E). In particular, there was an increase of cholic acid and in 7α-hydroxy-4-cholesten-3-one (7-HCO), a biochemical intermediate in the rate-limiting reaction converting cholesterol to bile acids. The 7α,12α-dihydroxy-4-cholesten-3-one, an intermediate in cholic acid synthesis, showed a trend for increase (Fig. 5E, supplementary Fig. 4E, F). These results augment the finding that GRA induces an increase in cholesterol absorption and suggest that GRA mediates an increase of bile acid synthesis.
increases GLP-2, and modulates bile acid composition, as factors that could potentially contribute to the mechanism for increasing intestinal absorption of cholesterol.

**Effect of GRA on hepatic gene expression in hGCGR mice**

Based on these novel findings in clinical samples from T2DM patients who were treated with MK-0893, we investigated further effects of GRA on mRNA expression in hGCGR mice. As expected, GRA1 and GRA2 suppressed hepatic mRNA levels of glucose-6-phosphatase catalytic subunit (G6pc), phosphoenolpyruvate carboxykinase 1 (Pck1), and hexokinase 2 (Hk2), but increased glucokinase (Gck). However, we observed that the effects of GRA1 and GRA2 on gene expression in the pathway of cholesterol synthesis were marginal. Only HMG-CoA synthase 2 (Hmgcs2) was found to be slightly increased by both compounds (Fig. 4D). The lack of significant changes in mRNA levels for Pcsk9, sterol regulatory element-binding protein (Srebp)-1c, Srebp-2, and Ldlr is consistent with the limited impact of GRA1 and GRA2 on cholesterol synthesis (as ascertained isotopically in earlier aspects of this study) and a limited effect on components governing cholesterol clearance (Figs. 2E, 4B). On the other hand, the gene encoding Cyp7a1, which catalyzes the rate-limiting step of bile acid synthesis, was dramatically increased by GRA1 and GRA2 (Fig. 4D). Other genes controlling bile acid synthesis, Cyp27a1 and Cyp46a1, were also induced by GRA1 and GRA2, though to a lesser extent (Fig. 4D). In both acute and subchronic studies, GRAs had no effect on mRNA levels of intestinal Npc1l1 and Abcg5/g8, and they did not affect mRNA levels of liver Abcg5/g8 (Fig. 4D, supplementary Table 2).

**GRA-induced increase in plasma cholesterol is abolished by ezetimibe**

Based on the findings that GRA increases cholesterol absorption and that this is a mechanism-based effect, we next undertook a study of whether administration of ezetimibe, which acts to reduce cholesterol absorption, would effectively mitigate GRA-induced increases in LDL-c. hGCGR mice on chow diet were treated with vehicle and GRA1 in the absence or presence of ezetimibe for 9 days. Ezetimibe did not alter the target engagement or glucose-lowering efficacy of GRA1 (Fig. 6A). Ezetimibe alone had no effect on plasma cholesterol due to a large increase in the rate of cholesterol synthesis (supplementary Fig. 6), a compensatory effect previously demonstrated (38), and this was similar during coadministration with GRA. But its coadministration with GRA1 abrogated the 14% increase of plasma cholesterol observed in the GRA1 treatment arm (Fig. 6B). Ezetimibe, alone and combined with GRA1, strongly inhibited intestinal absorption of cholesterol as measured using orally administered D₆-cholesterol (Fig. 6C), thus eliminating the increase of 29% that was observed under GRA1 treatment. In these studies, it was again observed that despite a clear effect of GRA1 treatment to increase cholesterol absorption, little net effect on fecal cholesterol excretion was detected; whereas ezetimibe, alone

**Fig. 6.** Induction of GRAs on cholesterol is abolished by combined treatment with ezetimibe (Eze). Male hGCGR mice at 10–12 weeks of age were treated with vehicle, GRA1 (30 mpk), MK-0893 (30 mpk), and GRA2 (30 mpk) with or without ezetimibe (10 mpk) for 9 days (n = 8). A: Glucagon-induced glucose excursion after single dose on day 1. B: Plasma levels of total cholesterol. C: Cholesterol absorption calculated by the ratio of 2,2,3,4,4,6-D₆-cholesterol to 3,4-13C₂-cholesterol in plasma. N.D., nondetectable. D: Cholesterol excretion determined by the percent change in fecal total cholesterol on day 4 versus day 0. E: Plasma levels of glucose, total cholesterol, campesterol, and sitosterol after 9 day treatment. All data are shown as mean ± SEM. Statistical significance is calculated by unpaired two-tailed Student’s t-test. Asterisks denote statistical significance of the compound treatment groups compared with the vehicle group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
and in combination with GRA1, caused a marked increase in fecal cholesterol excretion (Fig. 6D).

This pattern of findings observed using GRA1 in combination with ezetimibe was then tested using both MK-0893 and GRA2, and highly similar results were obtained. MK-0893 and GRA2 increased plasma total cholesterol by 17 and 14%, respectively, together with significant increases in plasma campesterol and sitosterol. Ezetimibe alone and in combination with MK-0893 and GRA2 significantly and similarly reduced plasma campesterol and sitosterol, and in the coadministration arms, maintained plasma cholesterol at normal levels (Fig. 6E).

**DISCUSSION**

The impetus for these investigations of the effect of GRA on cholesterol homeostasis in hGCGR mice arose from clinical observations that dose-dependent increases in LDL-c occur in T2DM patients treated with the GRA, MK-0893. The 12 week clinical trial of MK-0893 achieved clear evidence that antagonizing endogenous glucagon can improve hyperglycemia in T2DM, attaining impressive efficacy on HbA1c and with minimal risk of hypoglycemia. Yet, the collateral finding of a dose-dependent statistically significant increase in LDL-c raises a concern of increased cardiovascular risk, despite substantial improvement in hyperglycemia.

This was therefore a “bedside-to-bench”-inspired translational study, undertaken to determine whether the rise in plasma LDL-c was restricted to MK-0893 or, instead, was more likely to be mechanism based. Because our data supported that the effect was mechanism based, we sought to determine among cholesterol synthesis, excretion, absorption, or plasma clearance of LDL-c, which mechanism was primarily responsible. The initial step was to identify an appropriate preclinical rodent model, a necessity because the GRA compounds had been optimized for potency against human GCGR and were much weaker against mouse GCGR, reflecting species differences in GCGR homology (11). The hGCGR mouse demonstrated elevation of plasma cholesterol with three structurally distinct GRA compounds and to an extent similar to the rise observed in clinical studies, establishing both that this was a suitable model and inferring that the rise in plasma LDL-c is mechanism based. Chiefl y on the basis of isotopic flux determinations of endogenous cholesterol synthesis and separately of intestinal cholesterol absorption, it was determined that GRA raises LDL-c by increasing cholesterol absorption. Bolstering this interpretation is the observation that GRA significantly increases plasma phytosterols, recognized biomarkers of intestinal absorption of cholesterol, and comparable to the increase of cholesterol absorption detected by isotopic methodology. Furthermore, these findings on plasma phytosterols in the hGCGR mouse enabled our studies to pivot back to bench-to-bedside, and by using archived samples from the clinical study of MK-0893, significant increases of phytosterols were observed during treatment with this GRA.

The concept that glucagon influences cholesterol metabolism has been previously reported, though with uncertainty as to the responsible mechanism. Reports have appeared in the literature since the 1950s, describing an association between glucagon action and cholesterol. For example, destruction of α-cells increased plasma cholesterol in rabbits (39) and dogs (40), while infusing glucagon prevented hypercholesterolemia in rats fed with a high cholesterol diet (41). LDL-c and the rate of cholesterol synthesis in a patient with familial hypercholesterolemia were reduced by portacaval shunt surgery, which was associated with marked increases of plasma glucagon and bile acids (42). Furthermore, glucagon infusion into humans profoundly reduced plasma levels of cholesterol (43). GCGR−/− mice were reported to have a significantly higher plasma LDL-c than WT mice (32). Plasma cholesterol was reported to increase in diet-induced obese mice treated with a monoclonal antibody inhibiting GCGR (44). In preclinical studies with MK-0893, there was not a prominent signal of increased plasma cholesterol that was noted (unpublished observations), perhaps simply reflecting the lesser potency of this compound for rodent compared with human GCGR. Regardless, with the hindsight afforded by the clinical trial data with MK-0893, it is clear that the published literature does contain precedents for the observation that GRAs can influence cholesterol metabolism.

The mechanism underlying the prior findings was not rigorously addressed although the findings were attributed to various causes, prominent among which is the hypothesis that antagonism of glucagon can increase cholesterol synthesis. There is a rationale that blocking glucagon signaling and, hence, increasing cAMP would release inactivation of HMGCR (27). In addition, it has been reported that glucagon suppresses plasma PCSK9 (45) and increases hepatic LDLR protein levels (46), presumably as a consequence of effects on the Srebp-2 pathway (45). Knockdown of GCGR in db/db mice significantly increased LDL-c, which was attributed to elevated hepatic lipogenesis and cholesterol synthesis (47). Therefore, in the current studies, it was initially evaluated whether GRA mediated an increase in cholesterol synthesis or might decrease LDL-c clearance. Our findings do not support either hypothesis. LDLR, PCSK9, and rates of cholesterol synthesis were not significantly changed during treatment with multiple GRAs, despite unequivocal induction of increased plasma cholesterol. Instead, our studies reveal the novel finding that there is a strong effect of GRAs to increase absorption of cholesterol. It appears that there are marked differences between GRA treatment and GCGR knockdown. While GRAs increased LDL-c and HDL-c, GCGR knockdown only increased LDL-c without any effect on HDL-c. Mechanistically, GCGR knockdown induced cholesterol synthesis (47), whereas GRAs induced cholesterol absorption without any effect on cholesterol synthesis. Though our studies did obtain some insights as to what may, in turn, contribute to increased absorption of cholesterol, it is acknowledged that there are a number of aspects of this novel...
hypothesis that will require further investigation. One factor might be the effect of GRAs to raise secretion of the gut hormone, GLP-2. It is known that glucagon, GLP-1, and GLP-2 are dramatically increased in GCGR"""" mice (33, 48). Plasma levels of total GLP-1 and GLP-2 were both significantly increased by subchronic treatment of GRA1, GRA2, and MK-0893 in hGCGR mice. Dramatic inductions of plasma total GLP-1 and GLP-2 were observed in T2DM patients chronically treated with MK-0893. GLP-2 has been reported to induce crypt cell proliferation and its effect on intestinal lipid absorption depends on nutritional status and presence of GLP-1 (34, 36, 37, 49). Although induction of GRAs on GLP-2 is predictable based on results from GCGR"""" mice, it appears that GRA does not mirror the effect of glucagon on cholesterol metabolism. This, however, does not negate the published findings of glucagon on the Srebp-2 pathway, including LDLR (46), PCSK9 (50), and cholesterol synthesis (29). The induction of GRA on GLP-2 is so dramatic that it might have masked the activation on the Srebp-2 pathway caused by blockade of GCGR, inasmuch as intestinal cholesterol absorption and hepatic cholesterol synthesis are reversely affected by each other (51, 52).

Another set of observations obtained in the present studies of GRA treatment that we posit to be fruitful for further examinations concerns bile acids. Bile acids play a critical role in facilitating intestinal absorption of hydrophobic lipids, notably including cholesterol and also, bile acids are derived from cholesterol and thus intimately associated with hepatic cholesterol metabolism. The archived plasma samples from the clinical study with MK-0893 were assayed for bile acid concentration and it was found that these levels were increased, including cholic acid and cholic acid precursors. Cholic acid supplementation enhances cholesterol absorption in humans (50). In GRA-treated hGCGR mice, a consistent finding is increased Cyp7a1 mRNA expression, the enzyme that is rate-limiting in bile acid synthesis. Consistent with an increased mRNA level of Cyp7a1 in hGCGR mice, its proximal product (7-HCO) was increased in T2DM during GRA treatment. It might also be considered that bile acids can stimulate secretion of GLP-1 and GLP-2 from L-cells via activating the bile acid receptor, TGR5 (53). There is resurgent interest in the role of bile acids in metabolic signaling, particularly in the context of diabetes mellitus (54), and the current findings indicate that glucagon agonism and antagonism can be influential in governing bile acid homeostasis.

Our studies indicate that the effect of MK-0893 to increase LDL-c derives mostly from a net increase in absorption of cholesterol, and that this is a mechanism-based rather than a compound-specific effect. This conclusion is based upon consistent findings in the hGCGR mice using three structurally diverse GRAs. Yet, while the data of our preclinical studies indicate a likely mechanism-based effect to increase plasma LDL-c via increased cholesterol absorption, we do recognize that the reported clinical findings, to date, with the effect of GRA on plasma LDL-c are inconsistent. In the clinical trials of LY2409021 (5, 6) and with ISIS-GCGRrx (7), no induction of LDL-c was reported. It remains uncertain why these findings may differ from what was observed in the clinical trial with MK-0893. Perhaps it is noteworthy that clinical trials of LY2409021 and ISIS-GCGRrx were not exclusively GRA monotherapy, as was the situation with the clinical dose-ranging study of MK-0893; instead, many of the T2DM participants in those studies were also receiving metformin treatment (5, 7). Metformin generally improves hypercholesterolemia in humans, although the mechanism is still not well-understood (55). Clinical studies using MK-0893 in combination with metformin treatment found it to have increased efficacy in reducing hyperglycemia and substantially mitigated the increase in LDL-c observed with GRA monotherapy in T2DM (26).

In keeping with a translational emphasis of the present studies, the effectiveness of ezetimibe to abrogate a GRA-induced increase in cholesterol absorption and plasma LDL-c was investigated. Ezetimibe, the molecular target for which is inhibition of NPC1L1, effectively abolished the increase in cholesterol absorption caused by GRA without interfering with efficacy in reducing hyperglycemia. It is possible that GRA-induced hypercholesterolemia could be mediated, in part, by an effect on Npc1l1 (56, 57). This remains to be fully characterized, but regardless, we describe that blocking Npc1l1 with ezetimibe in a rodent model effectively suppresses GRA-induced hypercholesterolemia. We postulate that coadministering GRAs and ezetimibe will work equally well, or better, in humans because, unlike the exclusive expression of Npc1l1 in intestine in mice, Npc1l1 is expressed abundantly in the liver and intestine in humans (58), and ezetimibe exerts its lipid-lowering effect by blocking Npc1l1 in both the liver and intestine in humans (59).

In summary, we used various pharmacological tools to study the major cause of GRA-induced hypercholesterolemia in mice and humans. Studies in mice demonstrated that increased plasma cholesterol was mainly attributable to cholesterol absorption. Consistent with this finding obtained in rodents, we report the novel finding that the increase of plasma LDL-c and cholesterol was associated with increases in plasma phytosterols in humans. With respect to potential mechanisms that mediate GRA-induced increase in cholesterol absorption, we observed that GRA-induced increases in GLP-2 and a trend of increase in bile acid concentrations in patients with T2DM and in the rodent model, together with mRNA expression of hepatic bile acid synthesis enzymes in mice. These are factors that can plausibly contribute to increased cholesterol absorption. Finally, in keeping with the translational emphasis of these studies, it was shown that ezetimibe effectively mitigated GRA-induced hypercholesterolemia in mice. Thus, combining GRA and ezetimibe may provide a feasible approach to mitigating MK-0893-induced elevation of cholesterol in patients with T2DM. Collectively, these results provide novel insights into the effect of glucagon and GCGR antagonism in governing cholesterol homeostasis.

The authors thank Taro Akiyama and Peter Stein for critically reading the manuscript and providing constructive suggestions. Doug Johns, Cai Li, Dan Kemp, Tom Roddy, Jing
REFERENCES


2. Unger, R. H., and A. D. Carrington. 2012. Glucagonocentric re-
structuring of diabetes: a pathophysiologic and therapeutic make-

Kauffman, and B. J. Goldstein. 2011. Efficacy and tolerability of MK-
0893, a glucagon receptor antagonist (GRA), in patients with type 2 diabetes (T2DM). Diabetes. 60(Suppl 1):A85.

4. Engel, S. S., M. Reitman, L. Xu, P. J. Andryuk, M. J. Davies, K. 
Kauffman, and B. J. Goldstein. 2012. Glycemic and lipid effects of 
the short-acting glucagon receptor antagonist MK-3577 in patients 

A. Hardy, and A. J. Levin. 2013. The glucagon receptor antago-
nist LY2490921 significantly lowers HbA1c and is well tolerated 
in patients with type 2 diabetes mellitus: a 24-week phase 2 study. 
Diabetologia. 56(Suppl 1):S391.

J. A. S. Gullatt, H. Loh, and M. A. Deeg. 2013. Short-term admin-
istration of the glucagon receptor antagonist LY2490921 lowers 
blood glucose in healthy subjects and patients with type 2 diabetes. 
Diabetes Obes. Metab. 17:414–422.

7. Morgan, E., A. Smith, L. Watts, S. Xia, W. Cheng, and S. Bhanot. 2014. ISIS-GCGRRX, an antisense glucagon receptor an-
tagont, caused rapid, robust, and sustained improvements in gly-
cemic control without changes in BW, BP, lipids, or hypoglycemia 
in T2DM patients on stable metformin therapy. Diabetes. 63(Suppl 
1A):LB28.

Collaborative Atorvastatin Diabetes Study (CARDS): multicentre 
cardiovascular disease with atorvastatin in type 2 diabetes in the 
USA. J. Lipid Res. 36:226–237.

for the isolation and purification of total lipides from animal tissues. 


back-to-back studies in the same subject. J. Lipid Res. 52:1420–1428.

and application of the dual-isotope plasma ratio method for the measurement of intestinal cholesterol absorption in the hamster. J. 
Lipid Res. 35:328–339.


analyses with the administration of labeled water. J. Lipid Res. 53: 
1223–1231.

Demonstration of diet-induced decoupling of fatty acid and cho-
lesterol synthesis by gene expression array and 2H2O quantification. 


17. Engel, S. S., R. Teng, R. J. Edwards, M. J. Davies, K. Kaufman, and 
B. J. Goldstein. 2011. Efficacy and safety of the glucagon receptor 
antagonist, MK-0893, in combination with metformin or sitagliptin 
in patients with type 2 diabetes mellitus. Diabetologia. 54(Suppl 
1):S86.


Akinsanya, S. F. Previs, T. P. Roddy, and D. G. Johns. 2015. In vivo effects of anacetrapib on prebeta HDL: improvement in HDL re-
modeling without effects on cholesterol absorption. J. Lipid Res. 56: 
2858–2865.


Lam, R. S. Lowe, M. E. Stepanavage, T. A. Musliner, J. C. Cohen, et al. 2010. Indices of cholesterol metabolism and relative respon-
siveness to ezetimibe and simvastatin. J. Clin. Endocrinol. Metab. 95: 
809–809.

blood glucose, hyperglycagomia, and pancreatic alpha cell hyper-
USA. 100:1348–1443.


Metab. 86:1759–1764.