Angptl4 does not control hyperglucagonemia or α-cell hyperplasia following glucagon receptor inhibition

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Abstract

Glucagon is secreted from α-cells in the pancreas and stimulates hepatic glucose output. Increased plasma glucagon levels and hepatic glucose production are key contributing factors to the development and progression of diabetes (1–3). For this reason, glucagon receptor (GCGR) antagonists have drawn a significant amount of attention as potential antidiabetic drugs. It is well established that GCGR deficiency or inhibition effectively lowers blood glucose in animal models of type 1 (4, 5) and type 2 diabetes (6–9). GCGR antagonism also blunts glucagon-stimulated glucose production in humans (10, 11) and lowers blood glucose in healthy individuals and persons with type 2 diabetes (12–15).

These studies have provided evidence for clinically significant improvements in blood glucose levels without displaying overt hypoglycemia. Additional preclinical studies also have revealed the existence of a feedback loop between GCGR in the liver and the α-cells in the pancreas. Specifically, it has been shown that inhibition of the glucagon signaling pathway invariably triggers hyperglucagonemia and α-cell hyperplasia. α-Cell hyperplasia has been observed in GCGR knockout mice (16), glucagon knockout mice (17), prohormone convertase 2 knockout mice (18), liver-specific GCGR knockout mice (19) and liver-specific Gαt knock out mice (20). Pharmacologic knockdown of hepatic GCGR using antisense oligonucleotides (21, 22) or administration of GCGR-blocking antibodies (6, 23) also increased α-cell mass in rodents. Furthermore, glucagon cell hyperplasia has been observed in patients with inactivating mutations in GCGR (24, 25).

The mechanism triggering pancreatic α-cell hyperplasia in response to glucagon signaling blockade has been studied previously. Solloway et al. (26) showed that GCGR antagonism increased plasma amino acid levels, which stimulated α-cell hyperplasia in an mTOR-dependent manner. However, another recent study claimed that Angptl4, an inhibitor of lipoprotein lipase-dependent plasma triglyceride metabolism (27), links GCGR inhibition to hyperglucagonemia and α-cell proliferation (28). Here we used overexpression studies and Angptl4−/− mice to determine whether Angptl4 promotes hypersecretion of glucagon and α-cell proliferation, as has been reported by Ben-Zvi et al. (28). We also measured circulating Angptl4 levels in humans dosed with a specific and highly efficacious GCGR-blocking antibody.

Results

Angptl4−/− Mice Show Normal Hyperglucagonemia and α-Cell Hyperplasia in Response to GCGR Blockade. We used a recently described fully human GCGR-blocking antibody (α-GCGR) (6) derived using VelocImmune technology (29, 30) to explore whether Angptl4 controls glucagon secretion and α-cell growth following GCGR inhibition. We treated chow-fed male Angptl4−/− and littermate control mice with GCGR or control antibody (10 mg/kg) for 22 d. The Angptl4−/− mice had normal blood glucose levels (190 mg/dL), which did not change with control antibody treatment (Fig. 1A). α-GCGR administration similarly lowered blood glucose levels in the Angptl4−/− and control mice (Fig. 1A). Body weight did not change in either of the treatment groups (Fig. 1B). α-GCGR promoted increased pancreas weight, which was similar in the Angptl4−/− (34%) and control mice.

Significance

Glucagon supports glucose homeostasis by stimulating hepatic glucose output. Inhibition of glucagon signaling has drawn much attention because of potential implications for diabetes treatment. It is well established that inhibition of glucagon signaling effectively lowers blood glucose but results in compensatory glucagon hypersecretion and expansion of pancreatic α-cell mass. It was recently proposed that Angptl4, an inhibitor of lipoprotein lipase-mediated plasma triglyceride clearance, links glucagon receptor inhibition to α-cell proliferation. Here we confirm that Angptl4 is a powerful regulator of plasma triglycerides, but not of hyperglucagonemia or α-cell hyperplasia. We observed an increase in plasma amino acids in humans following administration of a glucagon receptor-blocking antibody, confirming preclinical findings indicate that amino acids mediate the compensatory α-cell response.
Angptl4 Overexpression Does Not Increase Circulating Glucagon or α-Cell Hyperplasia in Mice. Hydrodynamic delivery of Angptl4 expression construct to livers of chow-fed mice resulted in high circulating levels of Angptl4 (Fig. 2A). Angptl4 overexpression for 14 d increased plasma triglycerides by 8.6-fold (Fig. 2B), but did not affect blood glucose or circulating levels of glucagon or insulin (Fig. 2C–E). α-cell mass and β-cell mass were measured at 22 d after the start of the study and were similar in Angptl4-overexpressing and control mice (Fig. 2F–H). Importantly, hydrodynamic delivery of α-GCGR antibody expression construct did not affect plasma triglyceride levels (Fig. 2B), but lowered blood glucose levels (Fig. 2C), increased plasma glucagon levels (Fig. 2D), and increased α-cell mass (Fig. 2F and G). No changes were observed in plasma insulin levels or β-cell mass (Fig. 2E, F, and H). The magnitude of change induced by the α-GCGR expression construct was comparable to that obtained with α-GCGR antibody treatment (Fig. 1), and served as a positive control for the
Angptl4 overexpression study. Consistent with the data in Angptl4−/− mice, these overexpression data do not support a role for Angptl4 in the regulation of α-cell function or growth, but do confirm its involvement in triglyceride clearance.

**Angptl4 Plasma Levels Do Not Change with α-GCGR Administration in Humans.** Administration of a single dose of α-GCGR antibody (0.3 and 0.6 mg/kg dose levels were combined) to human volunteers lowered fasting blood glucose by 13–14% for up to 3 d (Fig. 3A). The reduction in blood glucose was accompanied by a pronounced increase in plasma glucagon level (Fig. 3B), as well as a 60–67% increase in circulating amino acid levels (Fig. 3C). Importantly, plasma Angptl4 or triglyceride levels did not change with α-GCGR treatment (Fig. 3D and E). These data show that GCGR antibody blockade in healthy euglycemic humans produced the expected increase in circulating levels of glucagon and amino acids, without a change in Angptl4. These results support the preclinical findings indicating that Angptl4 is not a mediator of the feedback loop between GCGR inhibition in the liver and glucagon secretion in the pancreas.

**Discussion**

We report here that (i) Angptl4 is not required for improved glycemic control, compensatory glucagon secretion, or the α-cell growth response to GCGR blocking antibody treatment; (ii) Angptl4 overexpression did not increase plasma glucagon, increase α-cell mass, or improve glycemia; (iii) GCGR-blocking antibody increased plasma glucagon and amino acids in humans without affecting circulating Angptl4 levels; and (iv) Angptl4 is a negative regulator of plasma triglyceride levels.

Ben-Zvi et al. (28) reported that Angptl4 mediates compensatory glucagon secretion and increases α-cell proliferation in mice treated with a GCGR antagonist. This was supported by the observation that GCGR antagonism improved glycemia in Angptl4−/− mice without increasing plasma glucagon or α-cell proliferation (28). Angptl4 is a circulating factor produced primarily by the liver and adipose tissue. Accumulating evidence indicates that Angptl4 is important for the controlling the physiological fluctuations in lipoprotein lipase activity during fasting (27). Our results contradict the findings of Ben-Zvi et al. (28), showing completely normal glucagon hypersecretion and α-cell growth response in Angptl4−/− mice following GCGR blockade with a monoclonal antibody. We also demonstrate that humans dosed with the GCGR-blocking antibody had elevated glucagon levels but no change in plasma Angptl4 levels. Finally, the lack of involvement of Angptl4 in the regulation of α-cell function and proliferation is supported by our hydrodynamic-overexpression studies. In these studies, high circulating levels of Angptl4 were associated with a dramatic increase in plasma triglyceride levels but no changes in plasma glucagon levels, blood glucose levels, or α-cell mass.

We do not know the reason for the discrepancy between our data and the findings reported by Ben-Zvi et al. (28); however, an important difference between the studies is that we measured α-cell mass following 22 d of treatment with a highly potent and efficacious monoclonal antibody (6), whereas Ben-Zvi et al. measured...
α-cell proliferation at 7 d after administration of a GCGR peptide antagonist with low affinity (33, 34). Short-term GCGR antagonism has been shown to lower blood glucose without severe α-cell hypertrophy (35). We can exclude differences in diet, because we have observed pronounced α-cell hyperplasia in both chow-fed and high-fat diet-fed mice treated with the α-GCGR antibody (6). Similar to the finding reported by Ben-Zvi et al. (28) that Angptl4 controls glucagon secretion and α-cell mass, the same group previously made the initial claim that Angptl8 was the long-sought “betatrophin” that induces β-cell growth (36). That study was discounted by an independent group (37) and eventually by the original authors (36, 38).

It was recently shown that amino acids are responsible for the hyperglucagonemia and α-cell hyperplasia that occur on inhibition of glucagon signaling (26). Elevated circulating amino acid levels arise from reduced uptake and conversion of amino acids into gluconeogenic precursors in livers of mice with inhibited GCGR signaling (8, 26). We have confirmed and expanded these findings to humans, in whom we observed robust increases in plasma amino acid levels following the administration of α-GCGR. The increase in plasma amino acid levels following the administration of α-GCGR is sensed by mTOR, a central regulator of cell growth and proliferation in response to amino acids (39). Thus, accumulating evidence suggests that amino acid level in the liver and the α-cells in the pancreas is sensed by mTOR to maintain glucose homeostasis.

In conclusion, the present data do not support a role for Angptl4 in the control of α-cell function or growth. Our findings that plasma triglyceride levels were markedly increased following Angptl4 over-expression and reduced by Angptl4 deletion further suggest that triglycerides do not affect α-cells in the pancreas. Our data confirm that Angptl4 inhibition lowers plasma triglyceride levels, which may represent a therapeutic strategy for hypertriglyceridemia and could reduce the risk for coronary artery disease (40).

**Materials and Methods**

**Constructs.** A cDNA coding for a C-terminal MycMhex epitope-tagged human Angptl4 (G25-A160) was generated by PCR using the primers 5’-atcagcagcgcgttacagcaaatcgtcagggctgagcgcacagtctg-3’ and 5’-tagtgaattccattgggacgctggccgacattcatcctggc-3’, with a DNA plasmid clone harboring untagged human Angptl4 (UBC reference sequence NM_139314.2) as the template. The resulting cDNA was cloned into a mammalian expression vector pRc/RS, equipped with the Ror1 signal sequence (NM_001312690.1) and the C-terminal tag. The clone was confirmed by DNA sequencing. The expression and secretion were evaluated by Western blot analysis of transfected HEK293 cell culture medium using an anti-Myc antibody (Cell Signaling Technology).

**In Vivo Studies.** All procedures were conducted in compliance with protocols approved by the Regeneron Pharmaceuticals Institutional Animal Care and Use Committee. We previously described the in vitro and in vivo characteristics of α-GCGR (REGN1193), a potent monoclonal GCGR blocking antibody (6). This antibody was used in all studies reported in this paper. The GCGR antibody and isotype control antibody were diluted with sterile PBS.

**Angptl4**

We generated constructs for the production of the monoclonal α-GCGR antibody, REGN1193, cDNAs for the heavy and light chains were cloned into the pRc/RS vector, under the control of the human ubiquitin promoter (UBC reference sequence NG_027722.2). Expression was confirmed by analyzing culture media from transiently transfected CHO cells, using anti-Myc-HRP (Pierce).

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harvested and prepared for determination of α- and β-cell masses by histology. In a separate study, human Fc was overexpressed and its expression measured in six different cell lines. Human Fc was highly expressed in liver, but not in heart, kidney, lung, spleen, or WAT (Fig. S4). These data confirm that hydrodynamic DNA delivery is restricted to liver.

**Blood Chemistry for Mouse Studies.** Blood glucose was determined using ACCUCHEK Compact Plus blood glucose monitoring system (Roche Diagnostics). Plasma triglyceride levels were assayed in a Beckman Coulter UniCelDx 800 Synopticon Clinical System (Beckman Coulter). Plasma glucagon and insulin levels were determined using Mercodia glucagon and insulin ELISA. Plasma amino acid levels were quantified using the L-Amino Acid Quantification Kit (Sigma-Aldrich).

**Western Blot Analysis.** Here, 1.0 µl of plasma from each animal was resolved by SDS/PAGE using Criterion TGX 4–20% precast gel (Bio-Rad) under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with an anti-Myc-HRP-conjugated antibody (Cell Signaling Technology) and detected using an enhanced chemiluminescent detection system.

**Histology.** Pancreata were fixed in 10% neutral buffered formalin solution for 48 h and then embedded in paraffin. Two sections of the pancreas from each animal were stained with an anti-glucagon (REGN745, an α-glucagon monochonal antibody generated in-house) or an anti-insulin (Dako) antibody, and areas of glucagon- and insulin-positive cells were measured using Halo digital imaging analysis software (Indica Labs). The percent of glucagon- and insulin-positive areas in proportion to the whole pancreas area were calculated. α- and β-cell mass was calculated by multiplying the α- and β-cell areas for each animal by its corresponding pancreas weight.

**Expression Analysis.** Total RNA was purified from all samples using the MagMAX-96 for Microarrays Total RNA Isolation Kit (Life Technologies), according to the manufacturer’s specifications. Genomic DNA was removed using MagMAXTurboDNase Buffer and TURBO DNase from the MagMAX mRNA Purification Kit (Invitrogen). Strand-specific RNA-seq libraries were prepared using KAPA mRNA-Seq Library Preparation Kit (Kapa Biosystems). Twelve-cycle PCR was performed to amplify libraries. Sequencing was performed using an Illumina HiSeq2000 Sequencing System by a multiplexed single-read run with 32 cycles. Raw sequence data (BCL files) were converted to FASTQ format using Illumina Casava 1.8.2. Reads were decoded based on their barcodes, and read quality was evaluated with FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were mapped to the mouse transcriptome (NCBI GRCh38) using ArrayStudio software (OlimicSoft), allowing two mismatches. Microarray analysis was performed as described previously (42).

**Human Studies.** Blood samples for glucose, glucagon, Angpt14, and triglycerides, and amino acids were collected from the 0.3 and 0.6 mg/kg dose groups as part of a single-center, phase I, single ascending dose, randomized, double-blind study to assess the pharmacokinetics, pharmacodynamics, safety, and tolerability of REGN1193. The full report of the study is in preparation. Each patient provided written informed consent, and the study was conducted in accordance with the International Conference on Harmonization’s Good Clinical Practice guidelines and all applicable local regulatory requirements and laws. Eligible subjects were healthy men and women, 18–45 y of age (inclusive), with a body mass index ranging from 18 to 30 kg/m² (inclusive), and with no history of change in body weight >10% over the 6 mo before screening. Other key inclusion criteria were hemoglobin A1C ≤5.5% and fasting plasma glucose 70–110 mg/dL.

After overnight fasting, blood was drawn for evaluation of glucose at baseline and on days 1, 2, 3, and 7 after i.v. administration of antibody or placebo and for evaluation of glucagon, total amino acids, Angpt14, and triglycerides at baseline and on days 2 and 7 after the administration. Glucagon levels were assessed using a validated assay at Pacific Biomarkers. Amino acids were measured using the L-Amino Acid Quantification Kit (Sigma-Aldrich). Angpt14 levels were determined using human Angpt14 Duoset ELISA (R&D Systems). Triglyceride levels were measured using a validated assay at Medpace Reference Laboratories. Data for the 0.3 and 0.6 mg/kg dose groups were combined for analysis.

**Data Analyses.** Data are reported as mean ± SEM. Statistical analyses were performed using Prism 6 (GraphPad Software). All parameters were analyzed by one- or two-way ANOVA; a threshold of P < 0.05 was considered statistically significant. If a significant F ratio was obtained, then post hoc analysis was conducted with Bonferroni posttests.

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Supporting Information
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Fig. S1. GCGR-blocking antibody promotes normal hyperglucagonemia and α-cell growth in Angptl4−/− female mice. (A) Fed blood glucose from chow-fed Angptl4−/− and control mice before and at multiple time points following s.c. injections of α-GCGR or control antibodies (10 mg/kg; n = 8/group). (B and C) Body weights (B) and pancreas weights (C) from the treatment groups described in A. The mice were killed on 22 d after dosing. (D–G) Plasma levels of triglycerides (D), glucagon (E), insulin (F), and total amino acids (G) from mice dosed as described in (A). (H–K) Representative immunohistochemistry images of pancreas section from a mouse from each of the four treatment groups stained for glucagon (H) or insulin (J), and α-cell mass (I) and β-cell mass (K) for the four treatment groups. Values are mean ± SEM. Statistical analysis was conducted by one- or two-way ANOVA with Bonferroni posttest. P values are comparisons to the Angptl4+/+ group. *P < 0.001; **P < 0.0001.
Fig. S2. GCGR-blocking antibody does not change Angptl4 expression levels in mice. Angptl4 expression levels were determined by RNAseq in liver, inguinal WAT (I-WAT), epididymal WAT (E-WAT), and BAT from chow-fed C57BL/6 mice given with α-GCGR or control antibodies (10 mg/kg; n = 4–6/group) once weekly for 21 d. Values are mean ± SEM. RPKM, reads per kilobase per million.

Fig. S3. Angptl4 expression is absent in liver and WAT of Angptl4<sup>−/−</sup> mice. Angptl4 expression levels were determined by microarray in liver and WAT from chow-fed Angptl4<sup>+/+</sup> and Angptl4<sup>−/−</sup> mice (n = 4/group). Values are mean ± SEM. A.U., arbitrary units.

Fig. S4. Hydrodynamic DNA delivery (HDD) is restricted to liver. Seven days post-HDD of human Fc (hFc) in chow-fed C57BL/6 mice (n = 4/group), hFc expression levels were determined by RNAseq in heart, kidney, liver, lung, spleen, and WAT. Values are mean ± SEM.