Glucagon-Like Peptide-1 Receptor Agonists Activate Rodent Thyroid C-Cells Causing Calcitonin Release and C-Cell Proliferation

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Liraglutide is a glucagon-like peptide-1 (GLP-1) analog developed for type 2 diabetes. Long-term liraglutide exposure in rodents was associated with thyroid C-cell hyperplasia and tumors. Here, we report data supporting a GLP-1 receptor-mediated mechanism for these changes in rodents. The GLP-1 receptor was localized to rodent C-cells. GLP-1 receptor agonists stimulated calcitonin release, up-regulation of calcitonin gene expression, and subsequently C-cell hyperplasia in rats and, to a lesser extent, in mice. In contrast, humans and/or cynomolgus monkeys had low GLP-1 receptor expression in thyroid C-cells, and GLP-1 receptor agonists did not activate adenylate cyclase or generate calcitonin release in primates. Moreover, 20 months of liraglutide treatment (at >60 times human exposure levels) did not lead to C-cell hyperplasia in monkeys. Mean calcitonin levels in patients exposed to liraglutide for 2 yr remained at the lower end of the normal range, and there was no difference in the proportion of patients with calcitonin levels increasing above the clinically relevant cutoff level of 20 pg/ml. Our findings delineate important species-specific differences in GLP-1 receptor expression and action in the thyroid. Nevertheless, the long-term consequences of sustained GLP-1 receptor activation in the human thyroid remain unknown and merit further investigation. **(*Endocrinology* 151: 1473–1486, 2010)**

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that promotes glucose-dependent stimulation of insulin and suppression of glucagon secretion (1, 2), delays gastric emptying (3), and reduces energy intake (4). GLP-1 also increases β-cell mass in preclinical studies via stimulation of β-cell proliferation and neogenesis and promotion of cell survival (5, 6). The rapid degradation of native GLP-1 by dipeptidyl peptidase-4 (7) has fostered the development of degradation-resistant GLP-1 receptor agonists with prolonged half-lives. These include exenatide (a GLP-1 mimetic administered as a twice-daily injection), exenatide once weekly (a microsphere-formulated version of exenatide), and liraglutide (a once-daily human GLP-1 analog). The relative anti-hyperglycemic activity of the different GLP-1 receptor agonists is dependent on their potency and the duration of exposure (8, 9).

The actions of GLP-1 are mediated by a G protein-coupled receptor, for which only one receptor subtype is known and whose sequence is highly conserved in mammals (93% identity for rat and human GLP-1 receptors) (10). The main effects of GLP-1 are mediated by GLP-1 receptors in the pancreas, intestine, stomach, and the sen-

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Abbreviations: CI, Confidence interval; DAB, diaminobenzidine; GLP-1, glucagon-like peptide-1; ISH, *in situ* hybridization; ISLB, *in situ* ligand binding; LEAD, Liraglutide Effect and Action in Diabetes; PCNA, proliferating cell nuclear antigen.

sory and central nervous systems, although receptors are present in other tissues (11, 12). In rodents, GLP-1 receptors have been detected on parafollicular thyroid C-cells. Studies with rat thyroid C-cell lines and thyroid tissues have shown that activation of the GLP-1 receptor leads to calcitonin secretion (13–15), actions blocked by the GLP-1 receptor antagonist exendin(9-39) (14). Plasma calcitonin is a specific biomarker for both C-cell activation and increased C-cell number (16), and changes in calcitonin levels are used in the diagnosis of C-cell disease in humans (17).

During the development of liraglutide, the effects of long-term drug exposure were investigated in rodents and nonhuman primates. To understand the consequences of GLP-1 receptor activation in thyroid C-cells, we assessed the secretory and proliferative actions of liraglutide and other GLP-1 receptor agonists *in vitro* and *in vivo*. We also assessed plasma calcitonin levels in diabetic patients treated with liraglutide. Our data suggest that GLP-1 receptor activation in rodents but not primates produces C-cell activation and cell proliferation. These findings may have implications for understanding the biology of sustained GLP-1 receptor activation in the treatment of metabolic disorders such as diabetes and obesity.

Materials and Methods

Acquisition of tissues

We fixed fresh thyroid and pancreatic tissues in 10% buffered formalin for *in situ* hybridization (ISH) and immunohistochemistry studies or froze the tissues in TissueTek Optimal Cutting Temperature compound for *in situ* ligand binding (ISLB) studies. We obtained samples of frozen and paraffin-embedded human thyroid glands from commercial suppliers after approval from local ethical committees and with written patient consent.

Test articles

Synthetic exenatide was a freeze-dried powder, 10 mg/vial (exendin-4, N-terminal amide, trifluoroacetate-salt; catalog no. H-8730; Bachem, Torrance, CA). We synthesized liraglutide, GLP-1, and other GLP-1 and exendin analogs at Novo Nordisk A/S (Bagsvaerd, Denmark).

ISH

ISH was carried out as previously described (18). The sense and antisense riboprobes were directed against the following sequences: mouse GLP-1 receptor (bp 383-987; EMBO AJ001692), rat GLP-1 receptor (bp 952-1285; EMBO S75952), human GLP-1 receptor (bp 136-658; GenBank NM_002062.2), rat calcitonin (bp 18-617; GenBank NM_017338), human calcitonin (bp 306-651; GenBank X00356), and mouse/rat/human cyclophilin (a 721-bp large DNA fragment; catalog no. 7375, DECAprobe; Ambion, Austin, TX). We applied autoradiographic emulsion and developed the sections after exposures of 3–5 d (calcitonin riboprobes), 20–25 d (GLP-1 receptor probes on mouse and rat pancreas or the cyclophilin riboprobe), and 40–50 d (GLP-1 receptor probes on monkey and human pancreata or thyroids from all four species).

ISLB

ISLB on tissue sections was performed essentially as previously described (12). Before ISLB assay, we demonstrated the presence of C-cells by calcitonin staining of every 10th section through six to 10 levels of the thyroid. Total binding used 0.3 nM [^{125}I]exendin(9-39) (PerkinElmer, Waltham, MA), and nonspecific binding was assessed with tracer plus 1 μM unlabeled GLP-1(7-37). We quantified the silver grains in sections with image analysis over C-cell-rich areas and expressed quantities as disintegrations per minute per milligram tissue by including a section of a commercial standard containing known quantities of ^{125}I.

Immunohistochemistry for calcitonin

Tissue sections were incubated with polyclonal rabbit antihuman calcitonin primary antibodies (catalog no. A0576; Dako Cytomation, Glostrup, Denmark) followed by biotinylated donkey antirabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; Dako Cytomation) and peroxidases (Dako Cytomation). We used diaminobenzidine (DAB) tetrahydrochloride or 3-amino-9-ethylcarbazole as substrates for visualizing the final peroxidase activity.

Immunohistochemistry for colocalization of calcitonin and GLP-1 receptor in C-cells

GLP-1 receptor polyclonal antibodies (K100B, K101B, and K102B) were generated by immunizing rabbits with an 18-amino-acid peptide (sequence TVSLWETVQKWREYRRQC) from the N terminus of the human GLP-1 receptor. We used polyclonal rabbit antihuman calcitonin primary antibodies (catalog no. A0576; Dako) as a marker for C-cells. Double staining of the GLP-1 receptor and calcitonin in the C-cells used a fluorochrome-labeled calcitonin method in sections processed with GLP-1 receptor staining using an avidin-biotin complex kit. We conjugated the polyclonal antihuman calcitonin rabbit antibody using the Alexa-488 conjugation kit (catalog no. A-20181; Molecular Probes, Eugene, OR). For details, see Supplemental Methods published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Cell line culturing

For details on cell line culturing, see Supplemental Methods.

Receptor binding assay

Receptor saturation whole cell binding assay is described in Supplemental Methods.

cAMP assay

cAMP accumulation was measured using the FlashPlate cAMP assay system (catalog no. SMP004A; PerkinElmer). For details, see Supplemental Methods.

Calcitonin release assay

Two days before experiments, we seeded 400,000 cells per well in Ham F12K (containing 0.92 mM Ca^{2+}) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in normal 24-well tissue culture plates. For the assay, cells were

cultured in DMEM with 15% horse serum and $CaCl_2$ to a final concentration of 3 mM and 200 U/ml aprotinin (catalog no. 616399; Calbiochem, San Diego, CA). For calcium sensitivity experiments, we used Ham F12K (containing 0.92 mM Ca^{2+}) with 15% horse serum and 1% penicillin/streptomycin, $CaCl_2$ to the desired final concentration, and 200 U/ml aprotinin. For calcium stimulation, we added a solution of $CaCl_2$ to give the final concentrations of Ca^{2+}. For details, see Supplemental Methods (calcitonin secretion *in vitro* and calcitonin assays).

Western blotting

We carried out Western blotting with denatured lysates after SDS-PAGE on precast 4–12% gradient gels. Proteins were electrotransferred to 0.45-μm polyvinylidene difluoride membranes. The antibody used for GLP-1 receptor detection is described in *Immunohistochemistry* and in Supplemental Methods, along with full details of the Western blotting.

Quantitative PCR

Cells were lysed with a guanidine hydrochloride buffer. Total RNA was extracted by acid phenol/1-bromo-3-chloropropane buffer followed by binding to silica particles. The level of GLP-1 mRNA was measured by real-time RT-PCR and evaluated relative to β-actin. Details of the method are described in Supplemental Methods.

Animal studies

Unless otherwise stated, we conducted animal studies with CD-1 mice (Crl:CD1; Charles River, Wilmington, MA) aged approximately 5–10 wk, Sprague Dawley rats (Charles River) aged approximately 6–7 wk, cynomolgus monkeys (*Macaca fascicularis*) aged approximately 1–2 yr, or GLP-1 receptor knockout mice lacking a functional GLP-1 receptor, aged 6–9 wk (Taconic M&B, Hudson, NY). Individual study designs are outlined in Supplemental Table 1, and details are given in Supplemental Methods. We conducted animal experiments following approved national regulations in Denmark and the United Kingdom and with animal experimental licenses granted by the Danish Ministry of Justice and the United Kingdom Home Office. We housed and handled animals according to current regulations, used environmental enrichment in all studies, and obtained local animal ethics committee approvals before study start.

Dosing

Unless otherwise stated, we injected animals sc and rotated between dorsal injection sites. For details, see Supplemental Methods.

Bioanalysis

We drew blood at prespecified time points from the ocular orbita (mice and rats), jugular vein (rats), or large limb veins (cynomolgus monkeys). We performed bioanalysis of plasma drug concentrations to evaluate drug exposure by validated RIA or ELISA. Val-Pyr/aprotinin was added to tubes to prevent enzymatic degradation.

Calcitonin analysis of plasma samples

We analyzed EDTA-stabilized plasma samples for calcitonin. For details, see Supplemental Methods.

Histopathological analysis

C-cell hyperplasia and tumor formation in response to GLP-1 receptor agonists was assessed in mice, rats, and cynomolgus monkeys (Supplemental Table 1). We prepared thyroid tissue sections according to standard histological procedures; diagnostic criteria from internationally recognized guidelines for preclinical histopathology were followed (19–22). In some studies, we used immunohistochemical staining for calcitonin to enhance the identification of C-cells (Supplemental Table 1). For determination of C-cell density (C-cells/mm^2) in the thyroid, C-cells stained by immunohistochemistry were counted by unbiased quantitative measurements carried out using the CAST grid system 3-axis VE (Olympus, Ballerup, Denmark). In the 104-wk rodent studies and the 4- and 13-wk monkey studies, we sampled one tissue section at the level of the parathyroid glands stained with hematoxylin and eosin. In the remaining studies, we sampled multiple thyroid sections. We applied a semiquantitative scoring system in the 16-month rat study. Here we scored focal C-cell hyperplasia on a scale from 0–2: 0, normal (0); minimal (1), one or two focal accumulations of C-cells the size of an average follicle; and slight (2), more than two focal accumulations of C-cells with one focus larger than an average follicle. We applied a quantitative scoring system in the 52-wk monkey study. Here, double immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and calcitonin was applied for the identification of proliferating C-cells. We estimated C-cell proliferation as the ratio between proliferating C-cells and the total number of C-cells. We also estimated the relative C-cell mass as the ratio between C-cells and follicular cells. To determine C-cell density, we counted C-cells/mm^2 in representative sections stained immunohistochemically for calcitonin.

Calcitonin mRNA analysis

Calcitonin mRNA levels were quantified in the thyroids of mice and rats after various treatments (Supplemental Table 1). We converted the RNA to cDNA and then used quantitative (TaqMan) PCR. Calcitonin mRNA levels were expressed relative to β-actin and GAPDH.

Calcitonin release in humans

Nine clinical trials of 20–104 wk duration were included in the calcitonin evaluation. Eight phase-3 trials in type 2 diabetic subjects [Liraglutide Effect and Action in Diabetes (LEAD) 1–6 plus two phase-3 trials in Japanese subjects] and one phase-2 trial in obese subjects. All trials were conducted in accordance with the Declaration of Helsinki; appropriate ethics committee approvals and informed patient consents were obtained before the start of each study. Calcitonin was evaluated by analyzing fasting calcitonin and calcitonin measured in a calcium stimulation test performed on a subset of subjects. For further details, see Supplemental Methods.

Statistical analyses

In vivo animal studies

We compared the calcitonin release values of different groups by estimating the ratio of their geometric means and 95% confidence interval (CI). We compared calcitonin protein synthesis data by estimating the ratio of their mean values and 95% CI and performing a Student's *t* test. We compared the incidences of C-cell hyperplasia with controls using a Fisher's exact test and

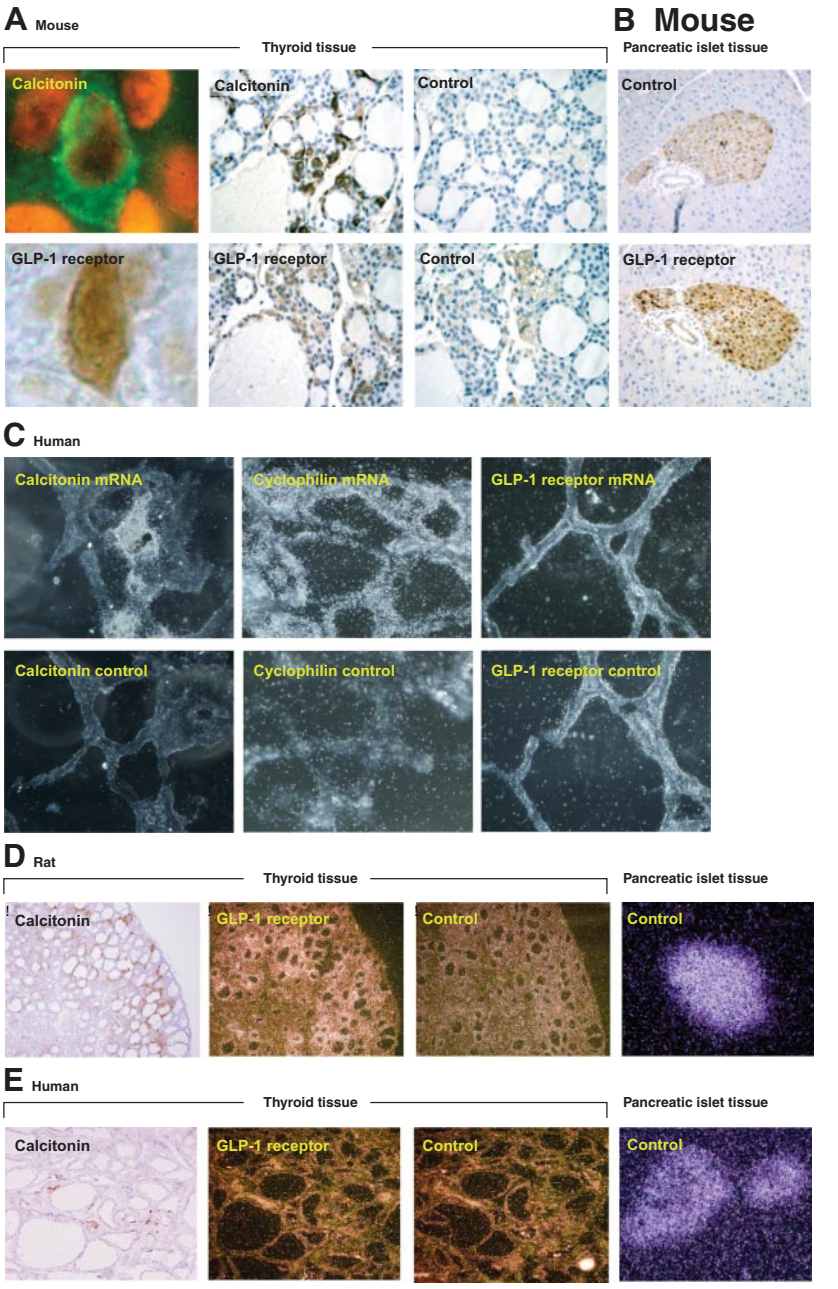

FIG. 1. The GLP-1 receptor was confined to thyroid C-cells in all species but not detectable by ISH or ISLB in human thyroid C-cells. A, Mouse thyroid C-cells after immunohistochemical staining for calcitonin [*top row*, *green* (*left*)/*brown* (*right*) staining] and the GLP-1 receptor (*bottom row*, *brown* staining). Control figures show no staining when omitting the primary antibody (*top*) and reduced cellular DAB reaction after preabsorption with the GLP-1 receptor peptide (*bottom*). B, Mouse pancreatic islet tissue showing GLP-1 receptor staining (*bottom*) and a reduced cellular DAB reaction after preabsorption with the GLP-1 receptor peptide (*top*). C, Human thyroid tissue after ISH for human GLP-1 receptor mRNA. *Bright silver* grains from autoradiography are shown by dark-field microscopy. The *top row*, when compared with corresponding controls on the *bottom row*, shows the presence of calcitonin mRNA and cyclophilin mRNA but no GLP-1 receptor mRNA. D and E, Rat (D) and human (E) thyroid tissue after immunohistochemical staining for calcitonin and ISLB with [^{125}I]exendin(9-39) in three adjacent tissue sections: calcitonin (*brown* DAB precipitate), GLP-1 receptor ligand binding (*bright silver* grains), control (competition with unlabeled GLP-1). The positive signal (difference between *middle* and *right* picture) for GLP-1 ligand binding occurred in all eight rats but not in any thyroid tissues from 13 human donors (seven postmortem donors and six fresh resected donors; females, n = 6, 45–76 yr; males, n = 7, 25–62 yr; two donor samples had C-cells localized in small clusters, eight had C-cells located as single cells, and three contained no C-cells). The final figure in each of D and E shows ISLB of [^{125}I]GLP-1(7-36NH$_2$) to rat and human pancreatic islets, respectively.

neoplastic changes, taking account of differences in mortality, assessed using Peto's model (23). The correlation between the early calcitonin response and the later development of focal C-cell hyperplasia in individual animals was analyzed by regression analysis. We analyzed the data from the 9-wk mouse study with a Wilcoxon rank sum test to compare the severity of C-cell hyperplasia, and ANOVA was used for data from other *in vivo* animal studies.

Clinical studies

Fasting calcitonin was analyzed by evaluating proportion of subjects increasing to 20 ng/liter or more (all trials). A repeated-measurement analysis for normal censored data with trial, treatment, and sex as fixed effects and patient as random effect was performed in the two 2-yr studies (LEAD 2 and 3). Endpoints from the calcium stimulation test were analyzed with an analysis of covariance with treatment and sex as fixed effects and baseline value as covariate. For further details, see Supplemental Methods.

Results

Thyroid C-cell number and GLP-1 receptor expression is species dependent

GLP-1 receptor expression on thyroid C-cells was identified by immunohistochemistry. Staining revealed the presence of calcitonin-immunopositive C-cells in thyroid tissue from mice (Fig. 1A), rats, cynomolgus monkeys, and humans (Supplemental Fig. 1, A–C); the GLP-1 receptor was localized exclusively to C-cells, as evidenced by colocalization of calcitonin and GLP-1 receptor immunopositivity. No other thyroid cell types exhibited GLP-1 receptor immunopositivity in any species, and controls demonstrated the specificity of the assays (Fig. 1, A and B).

C-cell densities were determined for mice, rats, and monkeys by computer-assisted cell counting in sections stained immunohistochemically for calcitonin. The mean ± SD C-cell densities in mouse and rat thyroid glands were 216 ± 62 and 449 ± 222 cells/mm^2 (n = 27–30, males and females combined), that is, 22- and 45-fold higher, respectively,

than that reported for humans (10 ± 26; n = 79) (24–26). The C-cell densities in thyroid glands from cynomolgus monkeys (23 ± 10; n = 6–8) and humans were comparable.

ISH using tissue from two human donors detected calcitonin mRNA in C-cells and cyclophilin mRNA in all thyroid cells (Fig. 1C). In contrast, GLP-1 receptor mRNA transcripts were not detected in human thyroid tissue. ISLB studies revealed GLP-1 receptor binding on rat C-cells (mean ± SE was 1699 ± 395 disintegrations/min · mg tissue; n = 8; Fig. 1D). In contrast, no signal was apparent in experiments using sections from thyroid tissues from 13 different human donors (representative experiment, Fig. 1E; Supplemental Fig. 1, D and E). Importantly, both rat and human pancreata had a good signal in agreement with β-cell GLP-1 receptor expression (Fig. 1, D and E).

Rat C-cell lines, but not human TT cells, express functional GLP-1 receptors

The acute functional effects of GLP-1 receptor agonists were explored in the rat C-cell lines rat MTC 6-23 and CA-77, which are known to express the GLP-1 receptor (13, 15), and a human C-cell line, TT cells (27).

GLP-1 receptor expression was quantified using three separate techniques: RT-PCR, Western blotting, and saturation ligand binding (Fig. 2, A–E). Levels of GLP-1 receptor mRNA transcripts were 14- to 21-fold lower in the human TT C-cell line compared with the rat C-cell lines (Fig. 2A). Western blot analysis revealed immunoreactive proteins corresponding to the predicted molecular weight of the GLP-1 receptor in the rat cell lines, whereas the human GLP-1 receptor was not detected in the TT cells (Fig. 2, B and C). The antibody was previously shown to recognize the human GLP-1 receptor (in immunohistochemistry, as shown in Supplemental Fig. 1C). Saturation binding experiments resulted in saturation binding curves with the expected affinity for GLP-1; a representative saturation plot from CA-77 is shown (Fig. 2D). The number of receptors expressed on the human cell line was determined by saturation binding analysis to be approximately 105 receptors per cell for the human cell line, whereas the number of receptors was 15- to 124-fold higher for the rat thyroid MTC 6-23 and CA-77 cells and the rat β-cell INS-1E cell lines (Fig. 2E).

We next assessed whether GLP-1 receptor expression in MTC cells was coupled to signal transduction and calcitonin secretion. Native GLP-1, liraglutide, and exenatide all potently elicited cAMP responses in the rat MTC 6-23 cell line. The relative potencies were higher for GLP-1 and exenatide than liraglutide (mean ± SD values for EC_{50}, 120 ± 70, 90 ± 40, and 5800 ± 2800 pM for GLP-1, exenatide, and liraglutide, respectively; n = 3 in all cases; Fig. 2F). These actions were GLP-1 receptor dependent because the GLP-1 receptor antagonist exendin(9-39) right-shifted the GLP-1-induced cAMP dose-response curve (Supplemental Fig. 2, A and B). In contrast, the forskolin-stimulated cAMP response was not antagonized by exendin(9-39) (Supplemental Fig. 2B). Moreover, forskolin, but not exenatide, GLP-1, or liraglutide, elicited an increase in cAMP levels in human TT cells (Fig. 2G).

Consistent with data from the cAMP experiments, all three GLP-1 receptor agonists elicited calcitonin responses in the rat MTC 6-23 cell line, with varying potencies (mean ± SD EC_{50} values were 80 ± 60, 55 ± 26, and 5300 ± 2400 pM, respectively, for GLP-1, exenatide, and liraglutide; n = 3 in all cases; Fig. 2H). Moreover, the stimulation of calcitonin secretion occurred via the GLP-1 receptor, because the GLP-1 receptor antagonist exendin(9-39) antagonized the effects of GLP-1, exenatide, and liraglutide (Supplemental Fig. 2C). In contrast, GLP-1 receptor agonists did not stimulate calcitonin release in human TT C-cells (Fig. 2I). To assess responses across the class of GLP-1 receptor agonists, experiments using taspoglutide and lixisenatide, structurally distinct GLP-1 receptor agonists, were performed. These compounds also potently elicited calcitonin release in a dose-dependent manner in rat MTC 6-23 cells (Supplemental Fig. 2D). Additional control experiments showed the rat and human C-cell lines responded to calcium with increasing calcitonin release, and GLP-1 potentiated the effect of calcium on calcitonin release in the rat C-cell line (Supplemental Fig. 2, E–G).

In rodents, GLP-1 receptor agonists stimulate calcitonin release in a GLP-1 receptor-dependent manner, followed by increased calcitonin gene expression

The designs of the *in vivo* studies are summarized in Supplemental Table 1.

Mice

Single liraglutide injections of 0.2–3.0 mg/kg induced an acute dose-dependent increase in plasma calcitonin that reached a plateau above 1.0 mg/kg · d [ratios of liraglutide to control (95% CI) were for 0.03 mg/kg · d, 1.09 (0.66–1.79), $P = 0.73$; for 0.2 mg/kg · d, 1.83 (1.11–3.01), $P = 0.02$; for 1.0 mg/kg · d, 2.36 (1.43–3.88), $P < 0.001$; and for 3.0 mg/kg · d, 2.43 (1.47–4.00), $P < 0.001$; ANOVA; Fig. 3A]. The increase in plasma calcitonin was dose proportionate; at the two highest doses, calcitonin levels remained elevated for more than 24 h (Fig. 3A). Calcitonin levels also rapidly increased immediately after administration of 0.25 mg/kg · d of exenatide [ratio of 0.25 mg/kg · d exenatide to control by continuous infusion (95%

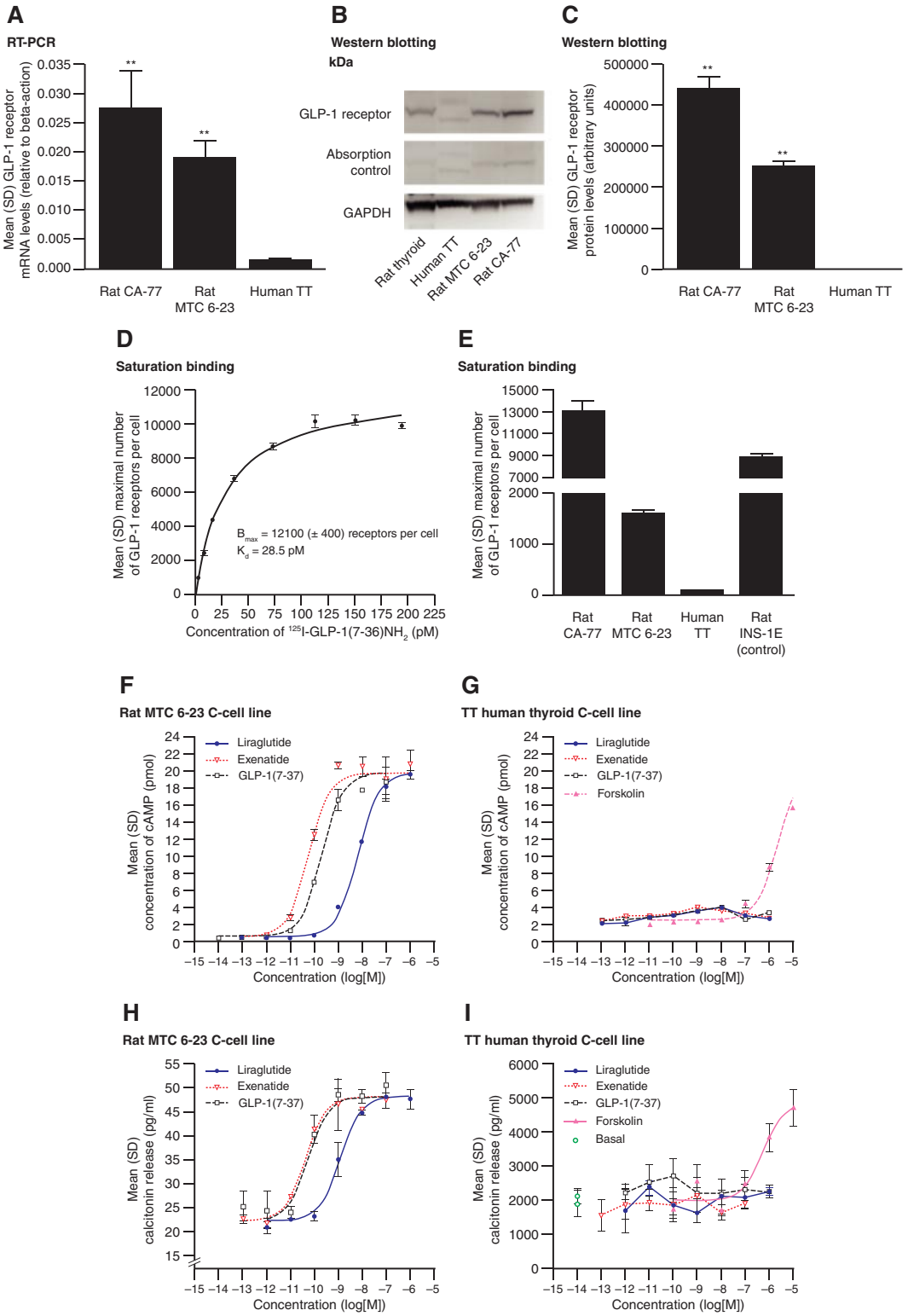

FIG. 2. The TT human thyroid C-cell line expresses few GLP-1 receptors compared with rat C-cell lines MTC 6-23 and CA-77 and shows a lack of functional response to GLP-1 and GLP-1 receptor agonists. A–E, Results of experiments assessing GLP-1 receptor and/or mRNA expression in C-cell lines; A, RT-PCR (n = 3); B and C, Western blotting analysis of GLP-1 receptor expression levels in rat and human cells (n = 3 for C). In B, lysate of rat thyroid was included as a positive control: *top*, GLP-1 receptor analysis (molecular mass 51 kDa); *middle*, adsorption control; *bottom*, GAPDH control. D and E, Saturation binding results (the GLP-1 receptor saturation curve shown in D is for the rat C-cell line CA-77; n = 2–3 for E). F–I, Functional responses of rat MTC 6-23 (F and H) and the TT human thyroid C-cell lines (G and I) to GLP-1 receptor agonists and forskolin in terms of cAMP accumulation and calcitonin release (n ≥ 3; data shown are from one representative experiment). **, $P < 0.01$ *vs.* human cell line (A, C, and E, *t* test). INS-1E is a rat pancreatic β-cell line (control).

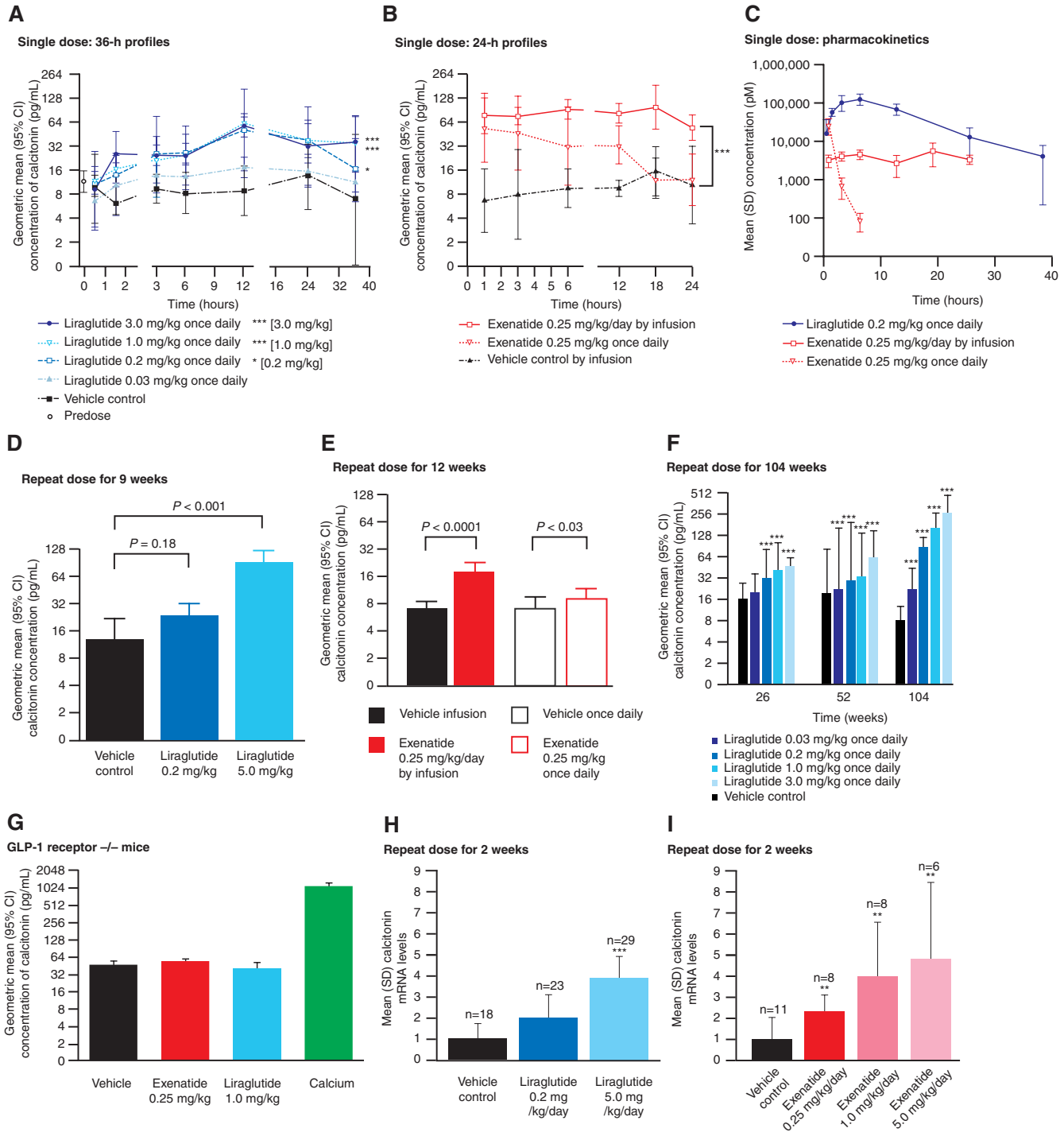

FIG. 3. Plasma calcitonin and calcitonin mRNA levels in CD-1 mice increased after dosing with GLP-1 receptor agonists. A and B, Plasma calcitonin was increased after a single liraglutide dose (A) and exenatide sc infusion (B). When the same total daily exenatide dose was given as a single sc injection, an increase was also seen during the first 12 h; after this, the response was similar to vehicle. C, Consistent with this, once-daily exenatide was relatively rapidly eliminated, whereas the liraglutide and exenatide infusion profiles were more comparable. Data are from two separate studies (liraglutide or exenatide). n = 5–6 per group per time point for A–C. D and E, Plasma calcitonin was still increased after 9 wk daily liraglutide dosing (D) and 12 wk exenatide dosing (E), again with a more pronounced increase with exenatide infusion than exenatide sc. F, Calcitonin increases progressed with time and dose with 104 wk repeat dosing of liraglutide. n = 8–36 per group per time point for D–F. G, Calcitonin levels in GLP-1 receptor −/− mice were not affected by dosing with liraglutide or exenatide (n ≥ 8 per group, sampled 6 h after dosing). Increased calcitonin levels were apparent in the positive control group (calcium; n = 4). Data for liraglutide are from a separate experiment with the same outcome for control groups. Calcitonin mRNA increased dose-dependently with liraglutide (H) (n = 18–29 per group) and exenatide (I) (n = 6–11 per group) for 2 wk. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$ for ratios of one treatment to another/vehicle unless indicated otherwise. Calcitonin mRNA levels are relative to β-actin/GAPDH and normalized against vehicle.

CI) was 7.96 (6.00–10.55); $P < 0.0001$, ANOVA; Fig. 3B]. Consistent with the shorter half-life of exenatide (*vs.* liraglutide) in mice (Fig. 3C), plasma calcitonin remained elevated after continuous infusion but fell rapidly after a single injection of exenatide (Fig. 3B).

Calcitonin levels remained elevated in mice treated with GLP-1 receptor agonists (9 wk liraglutide, 12 wk continuous exenatide, and 104 wk liraglutide dosing; Fig. 3, D–F). To determine the mechanism mediating the increase in calcitonin after administration of GLP-1 receptor agonists, we examined calcitonin levels in GLP-1 receptor −/− mice. Although plasma calcitonin levels were increased after administration of calcium, liraglutide and exenatide failed to increase plasma calcitonin levels in GLP-1 receptor −/− mice (Fig. 3G). In mice with functional GLP-1 receptors, both liraglutide (Fig. 3H) and exenatide (Fig. 3I) increased calcitonin mRNA levels in a dose-dependent manner.

Rats

Liraglutide increased levels of plasma calcitonin in rats (Fig. 4, A–C). After 4 wk liraglutide administration, calcitonin levels increased in a dose-dependent manner [ratio of liraglutide 0.75 mg/kg to control (95% CI) was 1.89 (1.49–2.41), $P < 0.001$; Fig. 4B]. During a 16-month study, calcitonin levels increased markedly in all dose groups (Fig. 4C). However, by 7 months, calcitonin levels were similarly elevated in control rats, consistent with published data (28). The calcitonin increase seen during the first 4 wk of treatment correlated, on an individual animal basis, with later development of focal C-cell hyperplasia ($P = 0.0012$). Mean calcitonin mRNA levels were 1.4-fold higher with liraglutide, 0.75 mg/kg · d, compared with vehicle after 4 wk dosing ($P > 0.05$; n = 14 per group, data not shown).

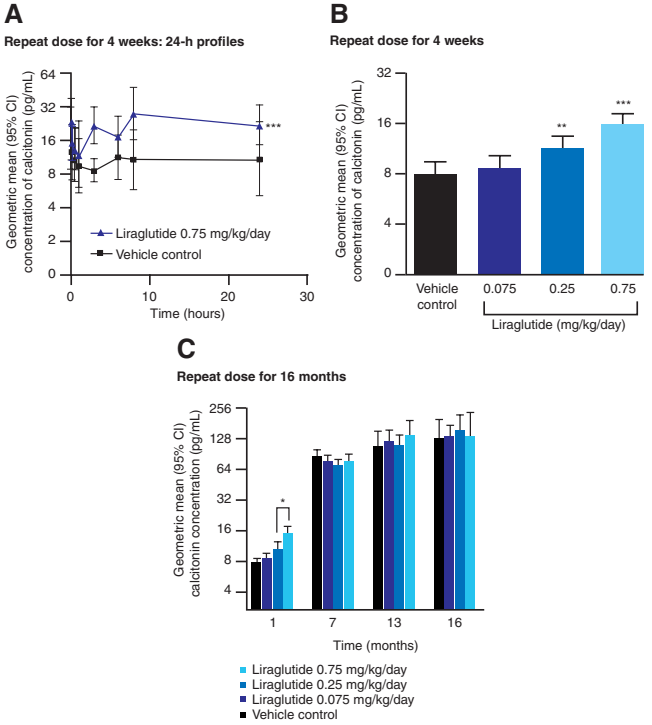

FIG. 4. Plasma calcitonin levels in Sprague Dawley rats were increased after dosing with liraglutide. A and B, A sustained increase in calcitonin levels was observed over 24 h (A) (n = 8 per group per time point) and a dose-dependent increase was seen 3 h after dose after 4 wk daily sc dosing (n = 40–44 per group) (B). C, Calcitonin increased over time in all groups during 16 months repeat dosing (n = 40–44 per group and 9–45 per time point, respectively). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with vehicle.

Long-term dosing with GLP-1 receptor agonists causes C-cell hyperplasia and tumor formation in rodents

Hyperplasia (Table 1)

Liraglutide administration was accompanied by C-cell hyperplasia after 9 wk dosing in mice (seven of 32 animals had hyperplasia with 5 mg/kg · d). Hyperplasia was reversed after cessation of treatment, as evident in the 15-wk recovery period. With exenatide, C-cell hyperplasia was similarly induced after 12 wk continuous infusion (11 of 36 animals had hyperplasia with 0.25 mg/kg · d). This effect was related to continuous drug exposure because the same total dose administered by single daily injection did not cause an increase in the incidence of hyperplasia. C-cell hyperplasia was generally more common in rats, because vehicle-dosed rats also exhibited spontaneous hyperplasia, whereas this was not seen in mice. In the 104-wk liraglutide studies, a dose-dependent increase in hyperplasia was seen in both rats and mice, with incidences reaching up to 54 and 38%, respectively.

Tumor formation (Table 2)

In 104-wk rodent studies, C-cell tumors increased after liraglutide administration in a dose-dependent manner. Rats treated with liraglutide had significantly more adenomas and carcinomas than control rats, whereas liraglutide-treated mice showed only significantly more adenomas than control mice. The only C-cell carcinomas in mice occurred in two females receiving a daily dose of liraglutide corresponding to an exposure that was 36-fold greater than the human dose.

Liraglutide does not cause calcitonin release or C-cell proliferation in nonhuman primates

In contrast to results in rodents, liraglutide had no effect on plasma calcitonin levels in cynomolgus monkeys after single doses or during 87 wk dosing (Fig. 5, A and B). A calcium challenge markedly increased calcitonin, but cal-

TABLE 1. Incidence of C-cell hyperplasia in mice and rats treated with GLP-1 receptor agonists

	GLP-1 receptor agonist								Vehicle control	
	M	F	M	F	M	F	M	F	M	F
Mice										
Liraglutide dose (mg/kg · d)			0.2	0.2			5.0	5.0		
Number of animals (n)			15–17	16–17			14–17	15–16	15–17	15–17
C-cell hyperplasia (%)										
At 2 wk			NA	NA			0	0	0	0
At 9 wk			6	6			6	38[b]	0	0
At 15 wk (6 wk recovery)			0	0			0	31	0	0
At 24 wk (15 wk recovery)			0	0			0	6[a]	0	0
Liraglutide dose (mg/kg · d)	0.03	0.03	0.2	0.2	1.0	1.0	3.0	3.0		
Number of animals (n)	66	66	65	67	67	66	79	76	79	75
C-cell hyperplasia (%) at 104 wk	0	0	2	10[b]	16[c]	15[c]	38[c]	29[c]	0	0
Exenatide dose (mg/kg · d)			0.25	0.25	1.0	1.0				
Number of animals (n)			12–18	12–18	12–18	12–18			12–18	12–18
C-cell hyperplasia (%)										
At 12 wk (continuous infusion)			33	28[a]	50[b]	58[c]			6	0
At 12 wk (single injection)			0	17					0	0
Rats										
Liraglutide dose (mg/kg · d)	0.075	0.075	0.25	0.25	0.75	0.75				
Number of animals (n)	49	49	50	49	50	50			50	50
C-cell hyperplasia (%) at 104 wk	29	29	40	55[b]	48[a]	48			22	28

Long-term dosing with GLP-1 receptor agonists causes C-cell hyperplasia in rodents. Statistical tests were Fisher's exact test for the incidence of C-cell hyperplasia and a Wilcoxon rank sum test in the 9-wk mouse study for the severity score for hyperplasia. M, Males; F, Females.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ *vs.* vehicle control for each sex except incidence of hyperplasia in female mice at 9 wk plus 15 wk recovery (24 wk), which was significantly reduced compared with incidence of hyperplasia in female mice at 9 wk.

citonin was not further increased by single or repeated-dose liraglutide (data not shown). Similarly, there was also no C-cell hyperplasia in chronic administration studies of 4, 13, 52, and 87 wk duration in monkeys (Supplemental Table 2). Representative thyroid gland sections, showing the absence of liraglutide-related effects on C-cell histology after 87 wk dosing are shown in Fig. 5C. Quantitative analysis of the thyroid glands from the 52-wk nonhuman primate study was performed using immunohistochemistry for C-cell detection and PCNA as a proliferation marker (Fig. 5D). No changes were observed in the relative thyroid C-cell mass (C-cell to follicular cell ratio) or proliferation index in the C-cells (Fig. 5, E and F).

Liraglutide does not cause calcitonin release in humans

Figure 6A shows estimated geometric means from the repeated-measurement analysis for the two studies with 2-yr data (1832 subjects were included in the analysis and 609 liraglutide-treated subjects completed the 2-yr trials). Estimated geometric mean levels remained around 1 pg/ml or lower at all time points in all treatment groups (estimated median baseline value, 0.59 pg/ml), well within the normal ranges for calcitonin (men, ≤8.4 pg/ml; women, ≤5.0 pg/ml; Fig. 6A). Furthermore, when combining data from nine clinical studies with durations of 20 wk or longer, there was no difference over time or between treatment groups in the proportion of patients whose calcitonin levels increased above a clinically relevant cutoff value of 20 pg/ml (Table 3). No women exhibited increases to calcitonin levels above 20 ng/liter. A subset of patients from two of the six phase-3 studies underwent a calcium stimulation test at baseline and wk 26 or 52 to assess functional C-cell activity. There were no significant differences for peak calcitonin levels or the ratio of peak to basal calcitonin levels between liraglutide (1.2 and 1.8 mg) and comparator treatments (Fig. 6, B and C).

Discussion

The present study shows that GLP-1 receptor agonists activate rodent thyroid C-cells, causing calcitonin release in a GLP-1 receptor-dependent manner. Furthermore, long-term GLP-1 receptor activation is associated with increased levels of calcitonin mRNA, C-cell proliferation, and tumor formation in rats and mice.

Liraglutide and four other GLP-1 receptor agonists (native GLP-1, exenatide, taspoglutide, and lixisenatide) all potently activated the GLP-1 receptor in rat thyroid C-cell

TABLE 2. Incidence of tumor formation in mice and rats treated with GLP-1 receptor agonists

	GLP-1 receptor agonist								Vehicle control	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Mice										
Liraglutide dose (mg/kg · d)	0.03	0.03	0.2	0.2	1.0	1.0	3.0	3.0		
Number of animals (n)	66	66	65	67	67	66	79	76	79	75
C-cell carcinoma (%)	0	0	0	0	0	0	0	3	0	0
C-cell adenoma (%)	0	0	0	0	13[c]	6[a]	19[c]	20[c]	0	0
Rats										
Liraglutide dose (mg/kg · d)	0.075	0.075	0.25	0.25	0.75	0.75				
Number of animals (n)	49	49	50	49	50	50			50	50
C-cell carcinoma (%)	8	0	6	4	14[b]	6			2	0
C-cell adenoma (%)	16	27[a]	42[b]	33[b]	46[c]	56[c]			12	10

Long-term dosing with GLP-1 receptor agonists causes tumor formation in rodents. Statistical tests used Peto's model for the incidence of neoplastic lesions.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ *vs.* vehicle control for each sex.

lines with the same maximal efficacy. Importantly, this activation was specific to the GLP-1 receptor because it was blocked by the GLP-1 receptor antagonist exendin(9-39). Consistent with a GLP-1 receptor-dependent mechanism, we did not observe calcitonin release with liraglutide or exenatide in GLP-1 receptor knockout mice.

We explored the possible relevance of the rodent findings by investigating human thyroid tissue, a human C-cell line, and a nonhuman primate model and by analysis of calcitonin data from clinical trials in patients with type 2 diabetes. Immunohistochemical staining detected GLP-1 receptors localized to C-cells in all species tested. Although GLP-1 mRNA transcripts colocalized with C-cells in the rodent thyroid gland (data not shown), no GLP-1 receptor signal was detected in the human thyroid gland by ISH. Furthermore, with ISLB studies, GLP-1 receptor binding in the rat thyroid was competed out with unlabeled ligand and was consistent with the presence of receptors on C-cells; GLP-1 receptor binding was not detected in human thyroid tissues.

We next investigated the functional effect of GLP-1 receptor agonists on C-cells using rat and human thyroid C-cell lines. The TT C-cell line has been used extensively as a model for studies of human C-cell biology (29–33). The studies using cell lines provided no evidence of a direct growth-promoting effect of GLP-1 receptor activation (Supplemental Information, Part 1), although proliferative effects were apparent in a pancreatic β-cell line. Although GLP-1 receptor agonists might theoretically stimulate β-cell proliferation as a mechanism for β-cell tumors (34, 35), we found no induction of β-cell tumors in pancreata of mice, rats, or monkeys in our studies (data not shown).

We used three different techniques to quantify GLP-1 receptor expression in rat and human C-cell lines and demonstrated that the number of receptors expressed in the human TT line was very low, and the corresponding signal for GLP-1 receptor mRNA was equally small. Importantly, whereas forskolin, the adenylate cyclase pathway activator, increased both cAMP and calcitonin levels in the human C-cell line, GLP-1 receptor agonists did not affect cAMP or levels of calcitonin in human TT cells.

Studies in mice and rats showed an increase in plasma calcitonin levels with GLP-1 receptor agonists. The calcitonin response was associated with increased calcitonin mRNA levels and, sequentially, by C-cell hyperplasia and tumor formation. In 2-yr studies involving wild-type mice, we observed dose-dependent C-cell hyperplasia and neoplasia, occurring only at doses that also caused increased calcitonin levels. In rats, we also reported a treatment-related elevation of calcitonin levels during the first month of dosing, which correlated with the later development of C-cell proliferation on an individual animal basis.

The C-cell stimulatory effects in rodents were more pronounced with continuous exposure to GLP-1 receptor agonists. These observations may have relevance for interpretation of comparable experiments carried out using second-generation GLP-1 receptor agonists designed to have enhanced potency and a more prolonged duration of action. We note that in previous 2-yr rodent carcinogenicity studies where tumors were seen in rats but not in mice, exenatide was administered at high doses but only once daily (36, 37). With the plasma half-life of exenatide in rats and mice, sustainable 24-h coverage in the rodents was likely not achieved. In contrast, we found a clear association between C-cell stimulatory effects in rodents and continuous exposure to GLP-1 receptor agonists. A single dose of exenatide did not stimulate prolonged calcitonin secretion, whereas the same total daily dose of exenatide administered continuously to mice elicited a magnitude and duration of calcitonin release that was the same as that for liraglutide. When dosed continuously, exenatide also

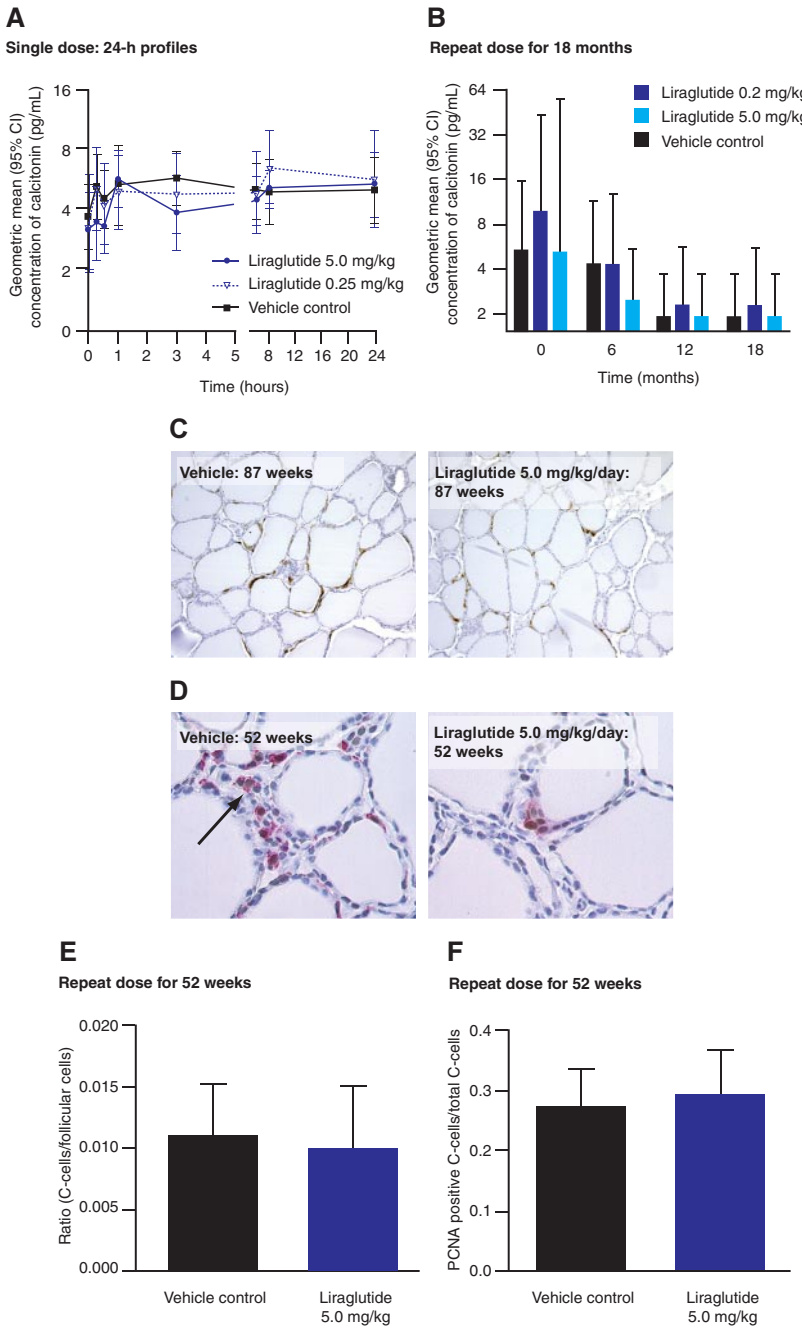

FIG. 5. Plasma calcitonin levels were not increased in cynomolgus monkeys dosed with liraglutide, and long-term dosing does not cause C-cell proliferation. A and B, No increase in plasma calcitonin was seen in cynomolgus monkeys receiving a single dose of liraglutide (A) or during 87 wk daily dosing (B). C and D, Representative thyroid gland sections from cynomolgus monkeys receiving vehicle or liraglutide 5.0 mg/kg · d for 87 (C) or 52 (D) wk. Tissues were sampled from the central part of the thyroid gland where the highest density of C-cells was found. Sections were stained immunohistochemically for the presence of calcitonin and counterstained with hematoxylin (*top row*) or double stained for calcitonin and PCNA and counterstained with hematoxylin (*bottom row*); ×50 or ×40 magnification is shown, respectively (*arrows* indicate cells double stained for calcitonin and PCNA). E and F, Liraglutide had no effects on the relative C-cell fraction of the thyroid gland (E) or C-cell proliferation as measured by the C-cell PCNA labeling index (F) after 52 wk treatment. n = 6–10 per group.

resulted in a similar frequency of C-cell hyperplasia in mice as liraglutide.

We also investigated whether liraglutide had any cross-reactivity to 75 different receptors or ion channels and found this not to be the case (Supplemental Information, Part 2). We also ruled out indirect augmentation of the calcium-induced stimulation of C-cells by liraglutide (Supplemental Information, Part 3).

Because nonhuman primates are genetically and physiologically more similar to humans than are rodents, we assessed liraglutide action in monkeys. In both mice and rats, C-cells are relatively abundant, and calcitonin is an important regulatory hormone in calcium homeostasis (38). C-cells are few in number in primates, however. Moreover, in humans, the physiological significance of calcitonin after birth is uncertain; calcitonin seems to have a major role in calcium homeostasis only in circumstances such as pregnancy or lactation (39). Although there are limited data available on C-cells in nonhuman primates, their calcitonin secretion in response to calcium is similar to that in humans (40). Interestingly, as little as 1 month treatment with vitamin D is associated with increased C-cell numbers in nonhuman primates (41), indicating that this species is an appropriate model for assessing proliferative effects on C-cells. In contrast, we did not detect increased calcitonin secretion or a proliferative response after liraglutide dosing for up to 87 wk in cynomolgus monkeys. Liraglutide doses provided exposures up to 60-fold greater than the highest dose recommended for the treatment of type 2 diabetes.

The relevance of the rodent findings was explored in humans via calcitonin monitoring in clinical studies. As shown in Fig. 6A, up to 2 yr exposure to liraglutide has not led to increased calcitonin levels. Geometric mean calcitonin levels remained within the lower end of the normal range and the patterns over time did not differ between liraglutide and active comparators. Importantly, there were no treatment-related differences in the number of patients moving above a clinically relevant cutoff of 20 pg/ml. Additionally, in the LEAD 6 study, which compared the efficacy and safety of once-daily liraglutide and twice-daily exenatide over 26 wk,

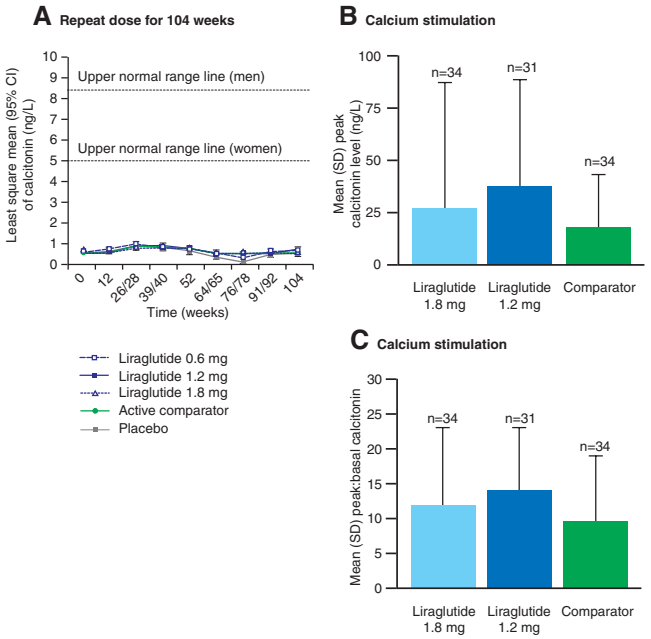

FIG. 6. Plasma calcitonin levels were not increased in patients with type 2 diabetes receiving liraglutide in phase-3 clinical trials. A, No increase in basal calcitonin levels was seen in the two longer-term phase-3 LEAD trials (LEAD trials 2 and 3 with respective extensions). Liraglutide did not significantly increase peak (B) or peak to basal (C) calcitonin levels *vs.* comparator after calcium stimulation (substudy for LEAD 3 and 4 main trials). Clinical data are from the safety populations and shown as least-square mean levels and pooled across studies. Data point at baseline in A is the median value pooled across treatment groups (0.59 pg/ml). Liraglutide was administered sc once daily in all studies.

neither compound was associated with increased calcitonin levels (9).

The absence of increases in calcitonin from human C-cells after liraglutide treatment implies that humans differ from rodents with respect to the GLP-1 receptor-mediated mechanism that can cause C-cell secretion and hyperplasia. Data from large population studies have validated the use of calcitonin as a specific and sensitive screening tool for evaluating C-cell status (42). Furthermore, in C-cell hyper- or neoplasia, both basal and stimulated plasma calcitonin levels increase (43). The normal calcitonin levels in patients treated with liraglutide are in contrast with increased calcitonin levels after treatment with the proton pump inhibitor omeprazole (44). Omeprazole and other proton pump inhibitors reduce gastric acid secretion and increase plasma levels of gastrin (45), which, in turn, lead to calcitonin secretion from the C-cells. Nevertheless, the long-term use of omeprazole is not associated with C-cell-related side effects in humans.

TABLE 3. Proportion of patients whose calcitonin levels increased above the clinically relevant cutoff value of 20 pg/ml

	Proportion of patients (%)		
	Liraglutide	Active comparator	Placebo
20–28 wk	0.3 (n = 3551)	0.2 (n = 1412)	0.1 (n = 710)
52 wk	0.2 (n = 1741)	0.3 (n = 630)	0.0 (n = 216)
104 wk	0.1 (n = 839)	0.3 (n = 320)	0.0 (n = 61)

The proportion of patients whose calcitonin levels increased above the clinically relevant cutoff value of 20 pg/ml was low and similar among treatment groups. All patients above 20 pg/ml were male. Data at wk 20–28 are from eight phase-3 diabetes clinical trials (LEAD trials 1–6 and Japanese trials 1700 and 1701) and one liraglutide obesity clinical trial. Data at wk 52 are from LEAD trials 2 and 3, Japanese trials 1700 and 1701, and the obesity trial. Data at wk 104 are from LEAD trials 2 and 3.

The significant species differences in C-cell responsiveness to GLP-1 receptor agonists in the present paper are consistent with not only species differences in the physiological roles of calcitonin but also with published data about the frequency and causes of C-cell proliferation in rats and humans. In rats, calcitonin is released to protect against postprandial hypercalcemia and, a link between the gastrointestinal axis and calcitonin secretion has been described as a mechanism that counteracts feeding-induced hypercalcemia (46). In line with this role in acute calcium regulation, the physiological half-life of calcitonin is very short (approximately 4 min) (47). The high frequency of C-cell proliferative lesions in rats observed in the studies reported here are consistent with previous observations demonstrating spontaneous incidences of C-cell hyperplasia in up to 75% of animals in 2-yr studies (48). Furthermore, increases in C-cell tumors have been reported with other pharmaceutical agents, such as the PTH analog teriparatide (49) and the calcium-regulating hormone vitamin D3 (50). We observed a greater incidence of C-cell hyperplasia after GLP-1 receptor activation in female mice. Gender-specific differences in C-cell biology have been previously described in several species, including humans (51). Moreover, C-cell hyperplasia and the spontaneous development of C-cell tumors are much more common in female than male rats (48). The precise mechanisms accounting for the enhanced spontaneous proliferation of rodent C-cells have not been elucidated.

In conclusion, we describe marked quantitative and qualitative species differences in GLP-1 receptor expression and function in the thyroid glands of rodents compared with primates. Our data demonstrate that exposure to a GLP-1 receptor agonist in rodents leads to calcitonin secretion, up-regulation of calcitonin mRNA, C-cell proliferation, and tumor formation. In contrast, we found no calcitonin release and no evidence of C-cell hyperplasia in monkeys after 20 months dosing of liraglutide at more than 60-fold the clinical exposure. Furthermore, calcitonin levels were not increased after 2 yr clinical exposure to liraglutide. Taken together, the data indicate that thyroid

C-cells in rats and mice differ markedly from primate cells in their response to GLP-1 receptor activation.

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L.B.K. and L.W.M. were involved in the generation, analysis, and review of data and wrote the paper; S.D.J. was involved in the analysis and review of data; S.A., K.A., A.S.d.B., C.G., F.E., A.C.H., H.J., A.M.M., H.S.N., J.N., and H.S. were involved in the generation of data; A.C.M., T.D.L.T., and M.Z. were involved in the clinical experiments; D.J.D. was involved in discussion of the data and in writing the article and provided the knockout mice.

Disclosure Summary: D.J.D. has served as an advisor or consultant within the past 12 months to Amylin Pharmaceuticals, Arena Pharmaceuticals Inc., Arisaph Pharmaceuticals Inc., Eli Lilly Inc., Glaxo Smith Kline, Glenmark Pharmaceuticals, Hoffmann-La Roche Inc., Isis Pharmaceuticals Inc., Merck Research Laboratories, Metabolex Inc., Novartis Pharmaceuticals, Novo Nordisk Inc., Phenomix Inc., and Transition Pharmaceuticals Inc. Neither D.J.D. nor his family members hold stock directly or indirectly in any of these companies. D.J.D. has received research grant support from Novo Nordisk for preclinical studies of liraglutide and the cardiovascular system. All other authors were employees of Novo Nordisk at the time the work was carried out, and most authors are minor shareholders in the company as part of an employee offering program. Novo Nordisk is developing liraglutide for the treatment of diabetes and obesity. Liraglutide is approved in Europe, USA, and Japan for treatment of type 2 diabetes.

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