RESEARCH

Secretion of parathyroid hormone may be coupled to insulin secretion in humans

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Abstract

Objective: Parathyroid hormone (PTH) is a key hormone in regulation of calcium homeostasis and its secretion is regulated by calcium. Secretion of PTH is attenuated during intake of nutrients, but the underlying mechanism(s) are unknown. We hypothesized that insulin acts as an acute regulator of PTH secretion.
Methods: Intact PTH was measured in plasma from patients with T1D and matched healthy individuals during 4-h oral glucose tolerance tests (OGTT) and isoglycemic i.v. glucose infusions on 2 separate days. In addition, expression of insulin receptors on surgical specimens of parathyroid glands was assessed by immunochemistry (IHC) and quantitative PCR (qPCR).
Results: The inhibition of PTH secretion was more pronounced in healthy individuals compared to patients with T1D during an OGTT (decrementalAUC$_{0-240min}$: −5256 ± 3954 min × ng/L and −2408 ± 1435 min × ng/L, $P = 0.030$). Insulin levels correlated significantly and inversely with PTH levels, also after adjusting for levels of several gut hormones and BMI ($P = 0.002$). Expression of insulin receptors in human parathyroid glands was detected by both IHC and qPCR.
Conclusion: Our study suggests that insulin may act as an acute regulator of PTH secretion in humans.

Key Words
- T1DM
- insulin receptor
- IGF1 receptor
- IGF2 receptor
- OGTT
- IIGI

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Introduction

In humans, parathyroid hormone (PTH) plays a major role in the regulation of bone turnover (1), but food intake has also been shown to play a role. Food intake acutely suppresses bone resorption in humans (2), a process which is probably coupled to the secretion of gastrointestinal hormones including glucose-independent insulinotropic polypeptide (GIP) (3) and glucagon-like petide-2 (GLP-2) (4). In support of this, administration of octreotide (a long-acting somatostatin analogue, which inhibits the secretion of the gastrointestinal hormones) significantly blunted the suppression of bone resorption after oral glucose in a study by Clowes *et al.* (5). However, in the

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same study, insulin was also inhibited by the somatostatin infusion which actually resulted in a concomitant increase in the plasma concentrations of PTH, raising the question that the reverse might also be true – a nutrient-induced insulin secretion might be coupled to inhibition of PTH secretion, thereby contributing to the nutrient-induced suppression of bone resorption.

We, therefore, hypothesized that insulin is an acute regulator of PTH secretion in humans. In order to elucidate this, we measured intact PTH in plasma from patients with type 1 diabetes (T1D) (confirmed C-peptide negative and, therefore, without residual insulin secretion) and from healthy individuals during an oral glucose tolerance test (OGTT) and during an isoglycemic i.v. glucose infusion (IIGI), respectively. Secondly, in order to isolate the possible effects of insulin on the regulation of PTH secretion, we designed a multiple regression model with the purpose of adjusting for the potential effects of several gastrointestinal hormones including GIP and GLP-2 known to affect bone resorption (6). Finally, we studied the expression of insulin receptors (*INSR*), insulin-like growth factor 1 receptors (*IGF1R*), and insulin-like growth factor 2 receptors (*IGF2R*) in the parathyroid gland on formalin fixed, paraffin embedded (FFPE) surgical specimens of the human parathyroid glands at the mRNA and protein level.

Materials and methods

Ethical concerns

The clinical study protocol was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration no. H-D-2008-037), registered with ClinicalTrials.gov (clinical trial no. NCT00704795), and performed in accordance to the principles of the Helsinki Declaration II (7). All participants gave written informed consent before inclusion. The immunohistochemical investigation of the FFPE tissue samples of human parathyroid glands extirpated accidentally during thyroid operations was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration no. H-17000290).

Subjects and study design

Reserve plasma samples from a previously published study (7) from our research group were used. The study population consisted, as described previously (7), of eight healthy, normal glucose-tolerant individuals (assessed by 75 g OGTT) without family history of diabetes and nine patients with T1D who all were C-peptide negative in response to a 5 g i.v. arginine test (subject characteristics are shown in Supplementary Table 1, see section on supplementary materials given at the end of this article). The participants had been subjected to 2 test days (a 50 g OGTT day and a IIGI day) separated by at least 48 h as previously described; patients with T1D took their long-acting insulin (insulatard, NovoMix or Levemir) the night before the experiment and were instructed not to take any insulin until after the experiments (7).

Biochemical measurement

Intact PTH was measured in plasma samples with Intellicheck-technology using VITROS Immunodiagnostic Products, ref. 680-2892 and 680-2893. 25-hydroxyvitamin D (25(OH)D) was measured in plasma (t=0 min) using a Liaison XL analyzer (DiaSorin, Saluggia, Italy). Insulin and C-peptide concentrations were measured using two-site assays (electrochemiluminescense, Roche/Hitachi Modular analytics, Roche Diagnostic) as previously reported (7). The detection limit for each assay is <2 pmol/L, and intra-assay coefficients of variation are 1.9% (insulin) and 4.6% (C-peptide) (8). Previously published data on GIP, glucagon, GLP-1 and GLP-2 (7) were also included in the current study to isolate insulin-dependent PTH regulation. Measurement of pancreatic and intestinal hormones are described elsewhere (7).

Parathyroid tissue samples

FFPE human tissue blocks from 13 accidentally removed parathyroid glands during thyroid surgery (all histologically normal but with no knowledge of diabetes status) were included in the study (Supplementary Table 2). A 2.0 mm recipient-punch was pressed 3 mm into the tissue block removing a tissue core from the parathyroid gland. RNA was isolated using the Qiagen RNeasy FFPE Kit (cat no.: 73504). RNA (1000 ng) was reverse transcribed using BioRad Superscript advance kit (cat no: 1725037).

IHC staining

Thirteen human parathyroid paraffin embedded tissue blocks were investigated by immunohistochemistry using antibodies against the INSR, IGF1R and IGF2R and paraffin embedded human kidney was included as control tissue. Four-micrometer-thick tissue sections were cut using a microtome and fixed onto coated glass slides. All tissue sections were dewaxed and pretreated by boiling

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in the microwave oven for 10 min in citrate buffer at pH 6 and subsequently pretreated with PBS containing 0.2% BSA. For the INSR staining, the tissue sections were incubated over night at 4 °C with the primary antibody diluted 1:200 (mouse anti insulin receptor, Abcam 36550). For visualization of the immunoreactions, the sections were incubated for 40 min with biotinylated goat anti mouse antibody 1:500 (Vector Laboratories, BA2000) as the second layer, followed by a preformed Avidin and Biotinylated horseradish peroxidase macromolecular complex (ABC) (code no. PK-4000, Vector Laboratories) for 30 min as the third layer. Finally, the immunoreactions were developed by incubation in 3,3-diaminobenzidine for 15 min. The sections were counterstained with Mayers Hemalum. For the IGF1R staining, tissue slides were incubated first with the primary antibody diluted 1:200 (mouse anti IGF1R, AMB7119, Biosite) over night at 4 °C and then on day 2 with biotin labeled goat anti mouse antibody, ABC complex and DAB as described previously for the INSR antibody staining. For the IGF2R staining, the primary antibody was diluted 1:400 (rabbit anti IGF2R antibody, LS B6310, Lifespan Biosciences), and on day 2 the secondary antibody used was biotinylated goat anti rabbit (1:500 Vector Laboratories, BA1000), followed by ABC complex and DAB as described previously.

Real-time polymerase chain reaction

Real-time PCR was performed on six tissue blocks (due to limited amount of parathyroid tissue after IHC staining only six tissue blocks were included in this part of the study). The expression of receptors was assessed by quantitative real-time PCR, using Qiagen QuantiFast SYBR (cat. no.: 204054) and Quantitect primers from Qiagen (*INSR* cat. no. QT00082810, *IGF1R* cat. no. QT00005831, *IGF2R* cat. no. QT00080549. Primer sets for *CASR* and *PTH* was used as positive control (cat. no. QT00055944, QT00008834). A primer set for thyroglobulin (*TG*) (cat. no: QT00095053) was used as negative control.

Statistical analyses

To assess distribution of data, residual plots and histograms were plotted, and Shapiro–Wilk tests for normality (Swik command) and Brown–Forsythe tests for variance within groups were performed. Area under curve (AUC) was calculated using the trapezoidal rule (40 min: $AUC_{0–40min}$ and 240 min: $AUC_{0–240min}$) adjusting for the mean of the three baseline values: −15, −10 and 0 min. Decremental AUCs (dAUC) were calculated for the PTH response and incremental AUCs (iAUC) were calculated for insulin. Non-parametric test was used to assess statistical differences between groups and intervention: Differences between OGTT and IIGI within groups were evaluated using a Wilcoxon Signed Rank test and across groups using the Mann–Whitney *U*-test. Relative expression was calculated using the $2^{\Delta Ct}$ method, normalizing the expression of the gene of interest to the expression of PTH. A multiple linear regression was designed using changes (Δ) of PTH levels as dependent variable and BMI, GLP-1, GLP-2, GIP, glucose, glucagon, and insulin as independent variables. Calculations were made using GraphPad Prism version 6.04 and STAT14 (Boston, MA, USA). Figures were constructed in GraphPad Prism and edited in Adobe Illustrator (Adobe Systems Incorporated). $P<0.05$ was considered significant. Data are shown as mean ± S.D.

Results

There was no significant difference in plasma concentrations of PTH at fasting across the groups and study days (mean ± S.D.; 37 ± 16 ng/L (T1D-OGTT), 43 ± 20 ng/L (T1D-IIGI), 50 ± 24 ng/L (controls-OGTT), and 48 ± 17 ng/L (controls-IIGI); $P>0.15$). There was no significant difference in 25(OH)D between patients with T1D (51.4 ± 24.2 nmol/L) and healthy controls (37.6 ± 17.0 nmol/L; mean ± S.D.).

PTH secretion (illustrated as percentage of basal levels) was significantly inhibited 20 min after oral or i.v. administration of glucose in healthy individuals and in patients with T1D (Fig. 1A) ($P<0.05$). dAUCs (Fig. 1B) during the entire 240 min oral glucose experiment ($dAUC_{0–240min}$) or until nadir value (40 min) ($dAUC_{0–40min}$) were significantly greater for healthy individuals than for patients with T1D ($dAUC_{0–40min}$: −1049 ± 636 min × ng/L compared to −567 ± 270 min × ng/L, respectively, $P=0.043$). Similarly, for the first 150 min of the IIGI, secretion of PTH was more suppressed in healthy individuals compared to patients with T1D (Fig. 1A). In healthy individuals, oral glucose administration lowered PTH secretion more than i.v. glucose (Fig. 1A and B, $P=0.039$). Also in patients with T1D, there was a more pronounced decrease in PTH from 0–20 min during oral vs isoglycemic i.v. glucose. However, there was no significant difference in dAUC over the 240 min test period ($dAUC_{0–240min,IIGI}$ −3459 ± 2130 min × ng/L vs $dAUC_{0–240min,OGTT}$ = −2408 ± 1435 min × ng/L, $P=0.15$). Patients with T1D were all C-peptide negative. Insulin concentration increased significantly more (34,336 ± 7095 min × pmol/L vs 19,736 ± 4200 min × pmol/L) during the

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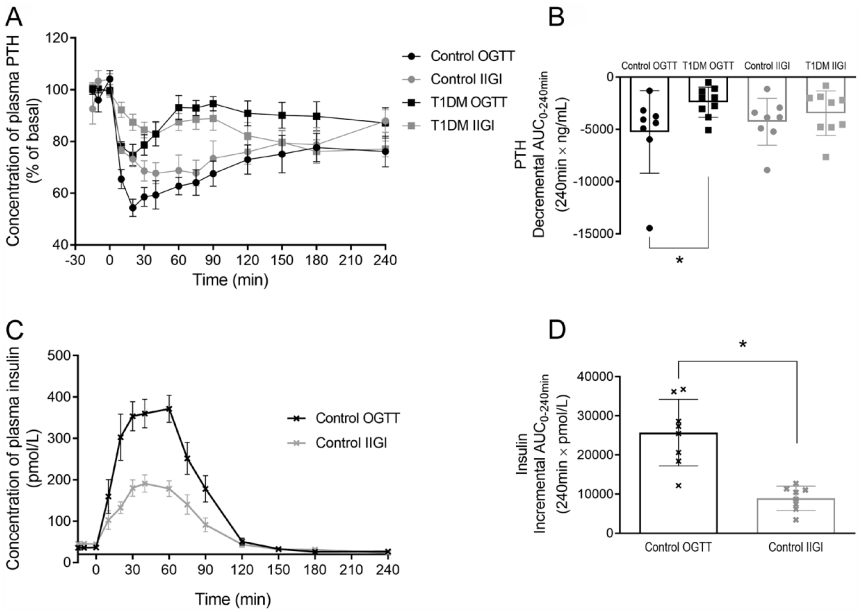

Figure 1
PTH secretion and insulin secretion during OGTT and IIGI in healthy individuals and in patients with type 1 diabetes. (A) PTH concentrations (percentage of basal) during an OGTT (black) or IIGI (grey) in healthy individuals (circles) and in patients with type 1 diabetes (squares). (B) Corresponding decremental area under the curve during the entire 240-min experiment ($dAUC_{0–240min}$) are shown. Individual data points are shown with mean and SD. (C) Insulin concentrations during an OGTT (black) or IIGI (grey) in healthy individuals. Corresponding $AUC_{0–240min}$ for C are shown in D. Individual data points are shown with mean and SD. For panel B, *represent statistical significant difference using an unpaired *t*-test, whereas for panel D, *represent statistical significant difference using a paired-test. Data are illustrated as mean ± S.E.M. *n* = 9/8 (T1DM/healthy individuals).

OGTT compared to the IIGI in the healthy individuals (Fig. 1C and D). Finally, in order to characterize the relation between insulin and PTH secretion further, we performed a multiple linear regression analysis of the entire dataset (Table 1), including plasma levels of GLP-1, GLP-2, GIP, glucose, 25(OH)D and glucagon. Insulin levels significantly predicted levels of PTH, also when correcting for GLP-1, GLP-2, GIP, glucose, 25(OH)D, glucagon, and BMI (P=0.006) (Table 2).

By immunohistochemistry, we found positive INSR staining primarily localized to the cell membranes of parathyroid chief cells in all parathyroid tissues analyzed and positive cell membrane staining for IGF1R and IGF2R staining in the majority of the tissue blocks. The most intense staining was found using the INSR antibody. Representative immunohistochemical stainings of parathyroid chief cells using antibodies against INSR, IGF1R and IGF2R are shown in Fig. 2A, B, C. Control staining of human kidney sections showed INSR and IGF-2R positive immunostaining in renal tubuli (Fig. 2D and F), in accordance with earlier published studies (9, 10). Human podocytes of the glomeruli were IGF1R immunoreactive (Fig. 2E), whereas the renal tubuli did not stain positively, in accordance with earlier published studies (11).

Analysis of mRNA isolated from FFPE parathyroid glands revealed expression of *INSR*, *IGF1R* and *IGF2R* as well as expression of *CASR* (Fig. 3). Two of the samples did contain traces of thyroid tissue as indicated by the expression of *TG*, but without any effect on the relative expression of *INSR*, *IGF1R*, *IGF2R* and *CASR* (Fig. 3).

Discussion

Our objective was to investigate the potential role of insulin as an acute regulator of PTH secretion. We performed

Table 1 Calculated AUC of hormones secreted during OGTT and IIGI.

Variables	Healthy individuals			Patients with type 1 diabetes		
	OGTT	IIGI	*P*-value[a]	OGTT	IIGI	*P*-value[a]
Insulin (min × pmol/L)	34,336 ± 7095	19,736 ± 4200	0.003	NA	NA	NA
GLP-1 (min × pmol/L)	2047 ± 360	1658 ± 336	0.01	2445 ± 483	1748 ± 324	0.001
GLP-2 (min × pmol/L)	3578 ± 824	3303 ± 903	0.03	4366 ± 1040	3655 ± 1152	0.002
GIP (min × pmol/L)	9180 ± 5892	2971 ± 2330	0.001	9299 ± 3864	2540 ± 1468	0.001
Glucagon (min × pmol/L)	1635 ± 614	1474 ± 476	0.570	1519 ± 386	1240 ± 257	0.117
C-Peptide (min × nmol/L)	318 ± 60	248 ± 39	0.015	0 ± 0	0 ± 0	NA
Glucose (min × mmol/L)	1400 ± 72	1397 ± 38	0.541	3986 ± 706	4147 ± 615	0.313

All data are given as mean ± SD. for $AUC_{0–240min}$. NA, not applicable. [a]Differences between oral glucose tolerance test (OGTT) and isoglycemic i.v. glucose infusion (IIGI). *P*-value based on two-sided paired *t*-test. Glucagon-like petide-1 (GLP-1), glucagon-like petide-2 (GLP-2), glucose-independent insulinotropic polypeptide (GIP), glucagon, C-peptide and glucose data from (7).

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Table 2 Multiple linear regression model of the relation between PTH and insulin in healthy individuals. Model 1: PTH as dependent variable and insulin as independent variable adjusting for GIP, GLP-1, GLP-2, glucagon, glucose, 25(OH)D and body mass index (BMI).

Model	R^2	Adjusted R^2	Number of observations	Probability of F (variance)	Degree of freedom	Root mean squared error
1	0.34	0.28	8	0.19	6	401
			Coefficients of the independent variables			
Model 1: dependent variables		Coefficient	S.E.	t score	Probability of t score	95% CI
Insulin		−0.019	0.007	−2.5	**0.002**	−0.033; −0.032
Glucose		−16.152	11.602	−1.11	0.315	−33.900; 7.325
GIP		−0.043	0.070	−0.61	0.565	−0.195; 0.107
GLP-1		1.898	1.981	0.78	0.460	−0.504; 3.920
GLP-2		−0.129	0.330	−0.38	0.712	−0.795; 0.547
Glucagon		−2.855	2.569	−0.66	0.556	−6.005; 1.0125
BMI		−7.678	34.632	−0.25	0.829	−78.728; 63.386
25(OH)D		0.212	0.125	0.95	0.81	−0.12; 0.328

analyses on plasma samples from both healthy individuals and C-peptide negative patients with T1D during OGTT and IIGI. We found significant and rapid decreases in PTH in the two groups during both test days. Using a multiple regression model, we found a significant relation between the postprandial rise in insulin and the decrease in PTH in healthy individuals, which was independent of other gastrointestinal parameters. We detected expression of *INSR*, *IGF1R* and *IGF2R* in parathyroid tissue using qPCR, and by IHC we were also able to localize INSR, IGF1R and IGF2R in parathyroid tissue. Our findings are, therefore, consistent with insulin as a physiological regulator of PTH secretion in healthy individuals; however, the preserved inhibition in the T1D patients shows that other mechanisms must also be operating.

A decrease in PTH after food intake has previously been demonstrated by several groups (5, 12, 13, 14, 15). However, due to the experimental protocol in these studies, it is impossible to decide whether insulin, the hyperglycemia, the combination or other factors were responsible for suppressing PTH after food intake. When adjusting for glucose in the multiple regression analysis of our data from healthy individuals, a significant correlation between insulin and PTH remained whereas no significant correlation was observed with glucose alone. In other words, the postprandial fall in PTH cannot solely be explained by hyperglycemia as also illustrated by the greater suppression during OGTT than during the IIGI in the healthy individuals, although glucose levels were identical. In further support, Christensen *et al.* (16) found a pronounced decrease of PTH from baseline during insulin-induced hypoglycemia in patients with T1D. These results are in accordance with results from Clowes *et al.* (17) and Fliser *et al.* (18), who showed a decrease in

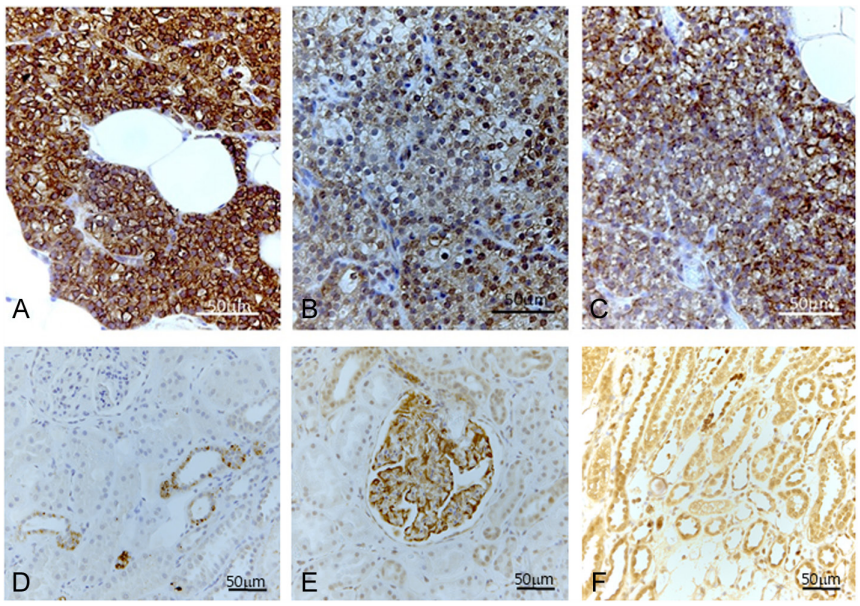

Figure 2
Immunohistochemical staining of human parathyroid gland showing INSR (A), IGF1R (B) and IGF2R (C) immunoreactive human parathyroid chief cells. Control staining of human kidney showing INSR (D) and IGF2R (F) immunoreactive renal tubuli and IGF1R (E) immunoreactive renal podocytes in renal glomeruli. Scale bar indicates 50 µm.

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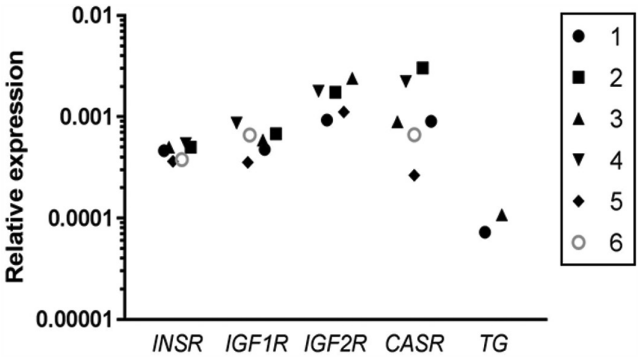

Figure 3
Relative expression of *INSR*, *IGF1R*, *IGF2R*, *CASR* and thyroglobulin (*TG*) found in the individual samples from FFPE human parathyroid tissue. Expression is normalized to the expression of *PTH*.

PTH secretion during hypoglycemic as well as euglycemic hyperinsulinemia in healthy individuals. Nevertheless, an insulin-independent effect of glucose is also evident from the two tests in patients with T1D, which showed an equal PTH suppression (because identical glucose levels were obtained) on the 2 days in spite of no insulin secretion. The marked suppression in healthy individuals during IIGI is, therefore, likely to represent a combination between an insulin-independent effect of glucose on PTH secretion and a direct effect of insulin secreted in response to the i.v. glucose.

It has been argued that the observed decrease in PTH level during hyperinsulinemic conditions could be due to elevated calcium levels (19). Several other studies, however, showed decreases in PTH during hyperglycemia/hyperinsulinemia that were independent of serum ionized calcium and total plasma calcium (14, 15, 17, 20, 21, 22). Changes in phosphate and magnesium levels following ingestion of glucose (23, 24, 25) could, at least in theory, also modulate secretion of PTH; however, evidence for this has not been reported. We did not measure calcium, phosphorus or magnesium which is a limitation of the study. To our knowledge, neither calcium levels nor phosphate levels differ between patients with T1D and healthy individuals, making it less likely that the significantly blunted inhibition of PTH secretion in patients with T1D is caused by these factors. Magnesium and vitamin D levels have been shown to be decreased in patients with T1D (26, 27) which could lead to reduced or increased PTH secretion, respectively. However, we found no difference in 25(OH)D between the groups in our study.

Valderas *et al.* (12) found a significant inverse relationship between bone resorption and insulin levels after a meal. This could be through a direct effect of insulin, as insulin receptors have been identified on both osteoblasts and osteoclasts (28, 29). With our identification of INSR in parathyroid tissue, we propose yet another association behind the inverse relationship between bone resorption and insulin involving a direct inhibitory effect of insulin on PTH secretion, supported by the significant inverse relationship between insulin and PTH secretion observed in our study. Our findings suggest that activation of this receptor may represent a target in the treatment of osteoporosis. Interestingly, we found a strong staining for the receptors in both oxyphil cells and chief cells. The function of the oxyphil cells in the parathyroid gland has not been identified; however, a study from Ritter *et al.* (30) suggested that oxyphil cells are derived from chief cells. Tanaka *et al.* (31) showed that, after heterotransplantation of human parathyroid nodules consisting exclusively of oxyphil cells or chief cells into nude mice, the mice were able to secrete intact human PTH in both situations. It is thus possible that the oxyphil cells also secrete PTH and that insulin also acts to suppress secretion from these cells. Our data do not allow us to draw any conclusions regarding the mechanisms involved in the insulin-induced inhibition, but we wondered whether paracrine mechanisms might be involved. Thus, in separate experiments, we searched for somatostatin receptor expression in parathyroid adenomas (which could be responsible for such an effect) but did not find any (N Borbye-Lorenzen and J Pedersen unpublished results).

Given the powerful effect of exogenous insulin to suppress PTH secretion (16), the larger inhibition observed during the OGTT day compared to IIGI day in the healthy individuals is likely to be due to higher insulin levels during the OGTT. In support of this, patients with T1D (and no insulin) had similar inhibition of PTH secretion on the IIGI day compared to the OGTT day ($P>0.10$).

The mechanism behind the insulin-independent suppression of PTH by glucose is unclear. We find it unlikely that glucose itself directly regulates PTH secretion but rather, as indicated in the study by Clowes et al. using somatostatin to block secretion of hormones, that the glucose-induced suppression of PTH secretion is coupled or mediated through an increased or decreased secretion of hormone(s) that we did not measure in this study. As we find expression of both *IGF1R* and *IGF2R* in the parathyroid glands, and as it has previously been shown that IGF1 and IGF2 stimulate PTH secretion (32), it is possible that the growth hormone (GH)/IGF-1 axis also is involved in the differential suppression of PTH following oral and i.v. glucose. GH secretion from the

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pituitary gland is known to be suppressed following oral glucose; however, the suppression of GH secretion is less pronounced when the glucose is delivered by the i.v. route (33, 34, 35), suggesting that the different suppression of PTH between oral and i.v. glucose observed in this study, in part, could be explained by differential suppression of GH and IGF1 secretion.

In conclusion, we here demonstrate the presence of INSR in human parathyroid cells. This, in conjunction with significant correlation between the increased insulin levels during oral and lower levels measured during i.v. glucose tolerance tests in healthy individuals and the lack of a similar difference in insulin-deficient patients with T1D, suggests that insulin may be involved in the acute regulation of PTH secretion. Thus, our results represent another piece of the nutrient-regulated bone turnover puzzle and this may lead to the identification of new targets for the development of bone disease therapies.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-20-0092.

Declarations of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution statement
MRS and BH planned and designed the study. KJH and FKK were responsible the clinical part of the study previously performed. KK provided the tissue sections. CØ and SSP performed the immunohistochemistry. JP and NBL performed the real-time PCR analyses. KJH, JJH, TV and FKK provided the plasma samples. NRJ performed PTH measurements. MRS, NJWA, JJH, CØ, SSP, JP and BH analyzed and interpreted data. MRS and NJWA drafted the manuscript. JP, NBL, KJH, TV, FKK, KK, CØ, SSP, NRJ, JJH and BH critically revised the manuscript for important intellectual content. All authors have provided approval of the final version to be published. BH is responsible for the integrity of the work as a whole.

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Disclosure Summary
The authors declare that they have nothing to disclose associated with this manuscript.

Clinical trials information
ClinicalTrials.gov (NCT00704795).

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