A novel oral dual amylin and calcitonin receptor agonist (KBP-042) exerts antiobesity and antidiabetic effects in rats

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Andreassen KV, Feigh M, Hjuler ST, Gydesen S, Henriksen JE, Beck-Nielsen H, Christiansen C, Karsdal MA, Henriksen K. A novel oral dual amylin and calcitonin receptor agonist (KBP-042) exerts antiobesity and antidiabetic effects in rats. Am J Physiol Endocrinol Metab 307: E24–E33, 2014. First published May 6, 2014; doi:10.1152/ajpendo.00121.2014.—The present study investigated a novel oral dual amylin and calcitonin receptor agonist (DACRA), KBP-042, in head-to-head comparison with salmon calcitonin (sCT) with regard to in vitro receptor pharmacology, ex vivo pancreatic islet studies, and in vivo proof of concept studies in diet-induced obese (DIO) and Zucker diabetic fatty (ZDF) rats. In vitro, KBP-042 demonstrated superior binding affinity and activation of amylin and calcitonin receptors, and ex vivo, KBP-042 exerted inhibitory action on stimulated insulin and glucagon release from isolated islets. In vivo, KBP-042 induced a superior and pronounced reduction in food intake in conjunction with a sustained pair-fed corrected weight loss in DIO rats. Concomitantly, KBP-042 improved glucose homeostasis and reduced hyperinsulinemia and hyperleptinemia in conjunction with enhanced insulin sensitivity. In DIO rats, KBP-042 induced a superior attenuation of diabetic hyperglycemia and alleviated impaired glucose and insulin tolerance. Concomitantly, KBP-042 preserved insulinostropic and induced glucagonostatic action, ultimately preserving pancreatic insulin and glucagon content. In conclusion, oral KBP-042 is a novel DACRA, which exerts antiobesity and antidiabetic efficacy by dual modulation of insulin sensitivity and directly decelerating stress on the pancreatic α- and β-cells. These results could provide the basis for oral KBP-042 as a novel therapeutic agent in type 2 diabetes.

TARGETING HYPERGLYCEMIA IN TYPE 2 DIABETES focuses primarily on improving insulin secretion and/or reducing insulin resistance (11, 37), although correction of hyperglucagonemia is equally important for optimal glycemic control (13). Furthermore, the majority of diabetic patients are overweight or obese, which contributes to insulin resistance and type 2 diabetes (20). Thus, optimally, novel antidiabetic drugs should improve all these parameters.

Glucagon-like peptide-1 (GLP-1) analogs (17) have demonstrated glucoregulatory effects through stimulation of insulin secretion, decreased glucagon secretion, and weight reduction (7).

Another therapeutic approach is to enhance insulin action and to avoid extensive hyperinsulinemia and increased insulin resistance (18, 42). Presently, insulin-sensitizing agents such as biguanides (e.g., metformin) and thiazolidinediones (e.g., glitazones) primarily reduce blood glucose but fail to reduce hyperglucagonemia and body weight. Additionally, the glitazones are associated with several adverse effects, including weight gain (1), highlighting the urge for novel therapeutic insulin-sensitizing agents.

The amylin analog pramlintide improves postprandial hyperglycemia by inducing glucagonostatic action, slowing gastric emptying, increasing satiation, and facilitating weight loss, thereby targeting several of the defects commonly seen in diabetic patients, although it lacks the ability to enhance insulin secretion and/or insulin action, and hence, is used solely as an adjunct to prandial insulin (12, 38, 44). These findings have led to a search for more potent amylin analogs with enhanced pharmacological properties (32, 33).

Two peptide hormones, salmon and eel calcitonin, are unique in their ability to activate both the amylin receptor and the calcitonin receptor with potencies that are superior to other ligands (4, 10). Recently, oral delivery of salmon calcitonin (sCT) demonstrated glucoregulatory efficacy by improved fasting and postprandial glycemic control, with a concomitant weight loss in diet-induced obese (DIO) rats (15, 51). Importantly, oral sCT attenuated diabetic hyperglycemia and preserved pancreatic β-cell function and mass in Zucker diabetic fatty (ZDF) rats (14). Hence, novel oral peptides with these abilities could be promising interventions in type 2 diabetes.

In this article, we present KBP-042, a novel dual amylin and calcitonin receptor agonist (DACRA) that was profiled in a series of in vitro receptor activation tests for the amylin, calcitonin, and the calcitonin gene-related peptide (CGRP) receptors. Finally, to establish proof of concept for in vivo efficacy of oral KBP-042, we performed a head-to-head study with oral sCT to explore the antiobesity and antidiabetic efficacy in DIO and ZDF rats.

RESEARCH DESIGN AND METHODS

Peptide Therapy

For oral delivery of sCT or KBP-042, the carrier agent N-(5-chlorosaliciloyl)-8-aminocaprylic acid (5-CNAC: vehicle) (21) was obtained from Biomics Biotechnologies (Nantong, China), and recombinant custom sCT or KBP-042 peptide (Unigene Laboratories, Boonton, NJ) was mixed with 5-CNAC (150 mg/kg). The doses chosen for sCT/KBP-042 peptide administration were based on previous studies in animal models of obesity and type 2 diabetes (14, 16) and in vitro settings (5).

In Vitro Receptor Binding and Activity

The relative receptor specificity and potency at the amylin and calcitonin receptor were determined by the ability of KBP-042 to induce cAMP, β-arrestin, and receptor binding in cell lines overex-
pressing the human calcitonin, amylin, and CGRP receptors, respectively.

Calcitonin and amylin receptor binding. U2OS CALCR cells and CHO K1 CALCR RPM3 cells (Path-Hunter β-arrestin Cell Line, DiscoverX; 93-0566C3 and 93-0268C2) were incubated with 250 pM (125I-(Tyr22)-sCT (NEX423; Perkin-Elmer, Waltham, MA) and unlabeled sCT (H-2260; Bachem, Bubendorf, Switzerland) or KBP-042 peptide (10⁻⁶–10⁻¹² M) for 30 min at ambient temperature. Cell lysate radioactive content was counted by scintillation using a 1470 Wallac Wizard γ-counter.

β-arrestin and cAMP quantification. U2OS CALCR cells, CHO K1 CALCR RPM3 cells (as described above), and CKO-K1 CALCRL cells (as described above) were used to quantify β-arrestin by PathHunter Detection Kit (DiscoverX; 93-0001) according to the manufacturer’s instructions. Cyclic AMP (cAMP) assays were conducted with 3-isobutyl-1-methylxanthine as a nonspecific inhibitor of cAMP phosphodiesterase. cAMP FEMTO TB KIT (62AM7PEB; Cisbio Bioassays, Codolet, France) was used to quantify cAMP content according to the manufacturer’s instructions.

Animal Experiments

All animal procedures were performed in accordance with guidelines from the Animal Welfare Division of the Danish Ministry of Justice under the institutional license issued to Nordic Bioscience (2012-15-2934-00094). Male selectively bred Sprague-Dawley DIO and male obese (fa/−fa) ZDF rats (Charles River Laboratories, Sulzfeld, Germany) were obtained at 5 wk of age and housed under controlled temperature on a normal 12-h light-dark cycle with unrestricted access to water and food. For DIO rats, chow consisted of regular rodent chow and a 60 kcal% high-fat diet (no. D12495; Research Diets) and was administered for a total of 12 wk. ZDF rats were fed Purina Laboratory Diet 5008 (Brogaarden, Lynge, Denmark) and allowed 1 wk of acclimation prior to experiments.

For short-term treatment, eight 17-wk-old DIO rats were assigned by body weight and glucose levels to receive oral administration of vehicle (150 mg/kg 5-CNAC) or oral KBP-042 (150 mg/kg 5-CNAC mixed with KBP-042 at doses of 0.5, 1, and 2 mg/kg) for 3 days (bid) using plastic feeding tubes (FTP-18-75; Instech Solomon, Plymouth Meeting, PA). Cumulative food intake and body weight were measured at study’s end, and oral glucose tolerance test (OGTT; 2 g/kg) was performed after overnight fasting.

For longer-term treatment, ten 17-wk-old DIO rats were assigned by body weight and glucose levels to receive oral administration of vehicle (5-CNAC), sCT, or KBP-042 at 1 mg/kg dose for a total of 4 wk (bidaily). Furthermore, a group pair-fed KBP-042 was included to assess the influence of food intake. Body weight and food intake were recorded weekly. To assess drug effect on gastric emptying, overnight-fasted rats received 96 mg/kg acetaminophen by oral gavage (4 ml/kg), and the appearance of acetalaminophen in plasma was monitored 30 min postadministration (32). Gastric emptying was calculated as percent change relative to vehicle: %change = 100 × [(peptide-plasma acetalaminophen/vehicle-plasma acetalaminophen) − 1]. At the end of the study, OGGT (2 g/kg) was performed in overnight-fasted rats with blood glucose measured and EDTA-plasma obtained for hormonal analysis. Homeostasis model assessment of insulin resistance (HOMA-IR) analysis was calculated and used for the estimation of hepatic insulin resistance using the formula (35) HOMA-IR = FI (µU/ml) × FBG (mM)/22.5, where FI and FBF represent fasting insulin and fasting blood glucose, respectively. Although HOMA-IR was developed for humans, it can be used as a surrogate measurement for insulin resistance in rodents (8, 25, 34).

For chronic treatment, 10 six-wk-old ZDF rats were assigned by body weight, Hb A1c, and glucose levels to receive oral administration of vehicle (5-CNAC), sCT, or KBP-042 (0.25, 0.5, 1, or 2 mg/kg) for 7 wk. Lean ZDF rats served as control. Fasting (12 h) and nonfasting blood glucose levels were measured, and Hb A1c was analyzed at the end of the study. OGTT (1 g/kg) (14) was performed after overnight fasting, and intraperitoneal (IPITT) (1 U/kg) and intravenous (IVITT) (0.4 U/kg) insulin tolerance tests were performed after 6 h fasting. For assessment of insulin sensitivity, calculation of the rate constant for blood glucose disappearance (Kitt) (3) was determined from blood glucose levels at 5, 10, and 15 min post-insulin injection during IVITT. Finally, animals were euthanized via an overdose of pentobarbital sodium (200 mg/kg iv) and decapitated. Pancreases were excised, homogenized, and extracted in acid-ethanol for subsequent determination of insulin and glucagon content (27). Protein content of these extracts was estimated by the bicinchoninic acid method (41).

Isole Isolation and Measurement of Insulin and Glucagon Release

Male lean healthy Sprague-Dawley rats (Taconic, Lille Skensved, Denmark) were obtained at 10–12 wk of age and islets isolated by retrograde collagenase solution injection via the bile-pancreatic duct (19). Isolated islets were hand-picked and transferred to incubation medium. Islets (4 islets·0.2 ml incubation buffer⁻¹·well⁻¹) were preincubated for 60 min at 37°C in 0.2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/l HEPES and 0.2% bovine serum albumin, as reported previously (2), which was modified to include 5.5 mmol/l glucose. After preincubation, identical buffer medium was exchanged, including 5.5, 11, and 22 mmol/l glucose for glucose-stimulated insulin-secreton (GSIS) or α-arginine (10 mmol/l, α-arginine hydrochloride 21%) (Sigma Aldrich Denmark, Copenhagen, Denmark) for arginine-stimulated glucagon-secretion with or without sCT or KBP-042 at a dose of 10⁻⁶ M and incubated for 60 min at 37°C. Immediately after incubation, aliquots of incubations/media were removed for analysis of insulin and glucagon, and the results were normalized to islet numbers.

Biochemical Analyses

Blood glucose was monitored by the Accu-Check Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland), and Hb A1c were monitored by DCA Vantage Analyzer (Siemens, Erlangen, Germany). Levels of insulin (Mercodia Rat Insulin ELISA; Mercodia, Uppsala, Sweden), glucagon (Glucagon Quantikine ELISA; R & D Systems Europe, Abingdon, UK), and leptin (rat leptin ELISA; Millipore, Billerica, MA) were analyzed according to the manufacturer’s instructions.

Statistical Analyses

All data are presented as means ± SE. The statistical analysis of drug effects vs. vehicle effects was conducted using one-way ANOVA, followed by Dunnett’s post hoc test. Student’s t-test was performed to compare lean control group and vehicle. All analyses were performed using GraphPad Prism software (GraphPad Prism, San Diego, CA). A value of P < 0.05 was considered to be significant.

Table 1. Amino acid sequence comparison of sCT, KBP-042, and eCT

<table>
<thead>
<tr>
<th>Sequence</th>
<th>sCT</th>
<th>KBP-042</th>
<th>eCT</th>
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<tbody>
<tr>
<td>HOMA-IR</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
</tr>
<tr>
<td>KBP-042</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
</tr>
<tr>
<td>eCT</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
<td>−CSNLSTCVLGKLSQELHKLTQYPRT−NH₂</td>
</tr>
</tbody>
</table>

sCT, salmon calcitonin; eCT, eel calcitonin. In addition, KBP-042 has an acetyl modification at the NH₂-terminal end for improved peptide stabilization. Letters in boldface are the same between KBP-042 and eCT; letters in italics are unique for KBP-042.
RESULTS

KBP-042 is a Superior and Specific DACRA

KBP-042 is an analog of both salmon and eel calcitonin, as the peptide sequence comparison listed in Table 1 illustrates. To assess activity on the amylin and the calcitonin receptor, we investigated ligand-mediated cAMP production, β-arrestin recruitment, and competitive ligand binding, using salmon calcitonin as comparators. KBP-042 activated both the amylin receptor and the calcitonin receptor, resulting in potent activation of both receptors (Fig. 1A, B, and C).

When compared with sCT, the potency of KBP-042 was found to be superior on these parameters (Table 2), apart from β-arrestin recruitment by the amylin receptor; however, the calculated EC₅₀ in the β-arrestin assay was affected by a significantly higher Eₘₐₓ for KBP-042 than for sCT (Fig. 1B). In the competitive binding studies, KBP-042 bound to both receptors with higher affinity than sCT (Table 2). Finally, no induction of the CGRP receptor was observed (Fig. 1C) (33).

Short-Term Oral KBP-042 Administration Improves Energy Balance and Glucose Tolerance in DIO Rats

Initially, we assessed the short-term metabolic effect of increasing doses of KBP-042 on energy balance and glucose tolerance during OGTT in DIO rats. During the treatment, KBP-042 induced a significant dose-related reduction in body weight (D), food intake (E), and glucose tolerance (F) and incremental area under the glucose curve (iAUC) during oral glucose tolerance test (OGTT) (G). n = 10 rats/group. **P < 0.01; ***P < 0.001. Statistical analysis between groups was evaluated by 1-way (D–F) and 2-way ANOVA (A–C and G) post hoc analyses. Data are means ± SE. RLU, relative light units.

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Fig. 1. KBP-042 is a specific in vitro dual amylin and calcitonin receptor agonist and improves energy balance and glucose tolerance during short-term treatment in diet-induced obese (DIO) rats. Dose range KBP-042 induction of β-arrestin in calcitonin receptor (CTR; A), amylin receptor (AMY-R; B), and calcitonin gene-related peptide receptor (CGRP-R; C) overexpressing cell lines. Short-term treatment effect of oral dose range KBP-042 in DIO rats with regard to body weight (D), food intake (E), and glucose tolerance (F) and incremental area under the glucose curve (iAUC) during oral glucose tolerance test (OGTT) (G); n = 10 rats/group. **P < 0.01; ***P < 0.001. Statistical analysis between groups was evaluated by 1-way (D–F) and 2-way ANOVA (A–C and G) post hoc analyses. Data are means ± SE. RLU, relative light units.

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weight (Fig. 1D) and food intake (Fig. 1E) compared with vehicle. Additionally, DIO vehicle rats demonstrated impaired glucose tolerance, which, in contrast, was markedly improved by KBP-042 treatment in a dose-related manner (Fig. 1F) and as evidenced by the 18, 39, and 46% decreases in the blood glucose incremental area under the glucose curve (iAUC) values for treatment doses of 0.5, 1, and 2 mg/kg, respectively (Fig. 1G) (15).

Longer-Term Oral KBP-042 Treatment is Metabolically Superior to Oral sCT in DIO Rats

To assess the antiobesity efficacy of KBP-042 in vivo, we investigated the effect of longer-term KBP-042 administration in DIO rats head-to-head with an equivalent dose of sCT and a pair-fed group for KBP-042 to explore the impact of food restriction on weight and glucoregulatory actions. KBP-042 and sCT (1 mg/kg bid) were administered orally for 4 wk. Importantly, bidaily oral dosing with KBP-042 and sCT at 1 mg/kg resembled equivalent plasma exposure (data not shown). KBP-042, sCT, and pair-fed groups showed significantly lowered body weight and food intake compared with vehicle (Fig. 2A and B), and after 3 and 4 wk of treatment, KBP-042 was superior to sCT and its corresponding pair-fed group (Fig. 2A). Thus, throughout the intervention period, KBP-042 induced a significant hypophagic response that was superior to sCT (Fig. 2B). Additionally, at 3 wk of treatment, the rate of gastric emptying was slightly increased in pair-fed rats (2.6% not significant) but significantly decreased in KBP-042-

### Table 2. *In vitro receptor binding and activity of sCT and KBP-042*

<table>
<thead>
<tr>
<th>Ligand</th>
<th>CTR (EC50, 10^{-10} M)</th>
<th>β-Arestrin (EC50, 10^{-9} M)</th>
<th>Competitive binding (IC50, 10^{-9} M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCT</td>
<td>1.7 ± 1.3 (3)</td>
<td>6.7 ± 1.0 (3)</td>
<td>3.9 ± 1.1 (3)</td>
</tr>
<tr>
<td>KBP-042</td>
<td>0.8 ± 1.5 (3)*</td>
<td>4.8 ± 1.0 (3)**</td>
<td>3.2 ± 1.2 (3)</td>
</tr>
<tr>
<td>Fold difference (KBP-042/sCT)</td>
<td>2.2</td>
<td>1.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; values in parentheses are the no. of individual experiments performed. CTR, calcitonin receptor; AMY-R, amylin receptor. In vitro screening data of sCT and KBP-042. EC50 values on cAMP production and β-arrestin recruitment as well as IC50 values from competitive binding with 0.25 nM 125I-sCT. All parameters measured on cells expressing human CTR or human AMY-R. In most instances, no difference in Emax for sCT and KBP-042 was observed. *P < 0.05, **P < 0.01, and ***P < 0.001, KBP-042 compared with sCT; †lower observed Emax for sCT compared with KBP-042.

Fig. 2. Longer-term administration of oral KBP-042 improves energy and glucose homeostasis and adiposity hormones in DIO rats. A: body weight during 4-wk intervention with oral doses (1 mg bid) of salmon calcitonin (sCT) and KBP-042. A group pair-fed KBP-042 (pair-fed) was included and dosed similarly to oral vehicle [N-(5-chlorosalicyloyl)-8-aminocaprylic acid, bid]. B: weekly food intake. C: change in fasting plasma glucose at the end of the study. D: homeostatic model assessment of insulin resistance (HOMA-IR). E and F: fasting plasma leptin (E) and plasma insulin (F) at the end of the study; n = 9–10 rats/group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. vehicle; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. sCT; †P < 0.05, ††P < 0.01, and †††P < 0.001 vs. pair-fed; §P < 0.05 vs. sCT (Student’s t-test). Statistical analysis between groups was evaluated by 1-way (B–F) and 2-way ANOVA (A) post hoc analyses. Data are means ± SE.
(−25.3 ± 1.8%, P < 0.001) and sCT-treated rats (−22.2 ± 2.1%, P < 0.001) compared with vehicle. Importantly, fasting blood glucose was modestly but significantly reduced for the KBP-042 group only, corresponding to improvement in indices of insulin resistance (HOMA-IR), compared with vehicle and pair-fed rats at the end of the study (Fig. 2, C and D). Interestingly, hyperleptinemia was significantly reduced in sCT- and KBP-042-treated rats compared with vehicle and pair-fed rats (Fig. 2E), whereas hyperinsulinemia was significantly reduced only in the KBP-042 rats compared with vehicle group (Fig. 2F), and no difference was observed between the KBP-042, sCT, and pair-fed groups.

Longer-Term Oral KBP-042 Treatment Improves Glucose Tolerance and Pancreatic Glucoregulatory Hormones in DIO Rats

To investigate the treatment effect of KBP-042 on glucose tolerance, we performed an OGTT at the end of the study. KBP-042 and sCT treatment markedly reduced blood glucose excursions following oral glucose exposure (Fig. 3A) and, to a similar extent, significantly reduced glucose iAUC values during OGTT compared with the vehicle and pair-fed groups (Fig. 3B).

To explore the glucoregulatory mode of action for KBP-042, we focused on pancreatic islet hormone response to glucose and demonstrated that (excessive) insulin secretion observed in the DIO and pair-fed groups was markedly suppressed during OGTT (Fig. 3C), and similarly, plasma insulin iAUC values were significantly reduced in KBP-042- and sCT-treated rats (Fig. 3D). Furthermore, KBP-042 and sCT treatment induced a glucagonostatic action (Fig. 3E) and significantly reduced plasma glucagon iAUC values during the initial period of the OGTT compared with the vehicle and pair-fed groups (Fig. 3F).

Chronic oral KBP-042 treatment exerts superior antihyperglycemic efficacy to oral sCT in ZDF rats.

To assess the antihyperglycemic efficacy of KBP-042 in vivo, we investigated the metabolic effect of chronic KBP-042 administration in ZDF rats with a head-to-head comparison with sCT. Multiple doses of KBP-042 and sCT (0.25, 0.5, 1, and 2 mg/kg bid) were administered orally for 7 wk. KBP-042 treatment at doses of 1 and 2 mg/kg and sCT at 2 mg/kg induced a significant vehicle-corrected gain in body weight (−8–10%) that was in line with previous findings (14). In ZDF rats, fasting and postprandial blood glucose levels decreased significantly over 7 wk by KBP-042 treatment at doses of 1 and 2 mg/kg (Fig. 4, B and D).

Fig. 3. Longer-term administration of oral KBP-042 improves glucose tolerance and pancreatic glucoregulatory hormones during OGTT in DIO rats. A and B: plasma glucose response (A) and iAUC values (0–240 min; B) during OGTT. C and D: plasma insulin response (C) and iAUC values (0–120 min; D) during OGTT. E and F: plasma glucagon response (E) and iAUC values (0–30 min; F) during OGTT; n = 9–10 rats/group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. vehicle; †P < 0.05, ††P < 0.01, and †††P < 0.001 vs. pair-fed. Statistical analysis between groups was evaluated by 1-way (B, D, and F) and 2-way ANOVA (A, C, and E) post hoc analyses. Data are means ± SE.
A and B), resulting in Hb A1c reduction by 1.6% at the end of the study (Fig. 4). Chronic administration of oral KBP-042 attenuates diabetic hyperglycemia and improves glucose tolerance in Zucker diabetic fatty (ZDF) rats. During OGTT, KBP-042 and sCT dose-dependently improved glucose tolerance at doses of 0.5, 1, and 2 mg/kg (Fig. 4, D and E), as evidenced by the significant decrease in iAUC during OGTT, which was reduced significantly by 50% at KBP-042 doses of 1 and 2 mg/kg (Fig. 4E). As observed above, KBP-042 treatment at a dose of 1 mg/kg exhibited a superior glycemic control compared with sCT (Fig. 4E).

**Chronic Oral KBP-042 Treatment Exerts Superior Pancreatic Glucoregulatory Effects on Oral sCT in ZDF Rats and Directly Modulates Insulinotropic and Glucagonostatic Action in Islets**

To explore the glucoregulatory mode of action for KBP-042, we focused on pancreatic-derived plasma hormones that were heavily involved in glycemic control. As expected, ZDF rats were severely hyperinsulinemic at baseline compared with the control rats (8.6 ± 0.4 vs. 0.3 ± 0.0 ng/ml, P < 0.001), reflecting an accelerating b-cell hypersecretion to compensate for the impaired insulin action. As observed previously (14), plasma insulin levels in the ZDF vehicle rats were progressively reduced during the study period, resulting in an ∼76% decrease at the end of the study. In contrast, KBP-042 treatment significantly sustained hyperinsulinemia at doses of 1 and 2 mg/kg, which in contrast was observed only for sCT at a dose of 2 mg/kg (Fig. 5A). As observed previously (14), fasting hyperglucagonemia was likewise a phenotypic trait in the ZDF rats compared with control rats (140.9 ± 8.9 vs. 57.9 ± 7.4 pg/ml, P < 0.001). In contrast, KBP-042 treatment at doses of 1 and 2 mg/kg significantly reduced plasma glucagon levels at the end of the study (Fig. 5B), which, as for insulinemia, was observed only at the 2 mg/kg dose of sCT. Notably, these insulinotropic and glucagonostatic effects would likely not be ascribed the incretin hormone GLP-1 (9), as KBP-042 treatment significantly decreased (normalized) plasma GLP-1 at doses of 1 and 2 mg/kg, which was observed only for the 2 mg/kg dose of sCT (data not shown), which is in line with previous findings (14). As expected in ZDF rats, pancreatic insulin content was significantly reduced at the end of the study compared with control rats (0.57 ± 0.03 vs. 1.38 ± 0.27 ng/g protein, P < 0.01). Importantly, KBP-042 at doses of 1 and 2 mg/kg significantly increased pancreatic insulin content compared with vehicle, which, in contrast, was observed only for sCT at the treatment dose of 2 mg/kg (Fig. 5C). Additionally, pancreatic glucagon content was significantly reduced in ZDF rats compared with control rats (55.86 ± 10.81 vs. 139.5 ± 16.27 pg/μg protein, P < 0.001) presumably as a result of pancreatic islet dysfunction and deterioration. Contrastingly, KBP-042 and sCT treatment at doses of 1 and 2 mg/kg preserved pancreatic glucagon content, although only at the KBP-042 dose of 2 mg/kg was statistical significance reached.
compared with vehicle (Fig. 5D). Finally, KBP-042, similarly to sCT, significantly reduced GSIS (Fig. 5E) and arginine-stimulated glucagon-secretion (Fig. 5F) in isolated islets from lean healthy rats.

**Chronic Oral KBP-042 Treatment Enhances Insulin Sensitivity Superiorly to Oral sCT in ZDF Rats**

To clarify whether KBP-042 preserved pancreatic function by alleviating insulin resistance, we performed insulin tolerance testing. We found that chronic treatment with KBP-042 and sCT led to significantly improved insulin tolerance for all doses tested during IPITT, which was illustrated with the highest dose of 2 mg/kg sCT and KBP-042 (Fig. 6A). However, because glucagonostatic action could influence counter-regulation and thus bias the true effect on insulin action, we compared blood glucose iAUC values during IPITT, and only KBP-042 doses at 1 and 2 mg/kg and sCT at 2 mg/kg were significantly reduced compared with vehicle (Fig. 6B). We confirmed that, during short-term IVITT, KBP-042 at doses of 1 and 2 mg/kg significantly enhanced insulin action and glucose disposal (Fig. 6C), with superiority to sCT at the 1 mg/kg dose.

**DISCUSSION**

Peptides with amylin-like properties, such as pramlintide (44) and, recently, davalintide (32), have shown promise as pharmacological interventions in obesity and type 2 diabetes; however, they are somewhat limited by poor efficacy in vivo.

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**Fig. 5. Chronic administration of oral KBP-042 modulates pancreatic glucoregulatory hormones in ZDF rats.** Fasting plasma insulin (A), fasting plasma glucagon (B), pancreatic insulin content (C), and pancreatic glucagon content (D) after 7 wk of multiple-dose (bid) oral sCT or KBP-042; n = 8–10 rats/group. Acute in vitro effect of KBP-042 and sCT (10 nM) on glucose-stimulated insulin secretion (E) and arginine-stimulated glucagon secretion (F) in isolated islets from lean healthy rats (8 different wells in 2–3 separate experiments). Statistical analysis between groups was evaluated by 1-way ANOVA post hoc analyses. *P < 0.05, **P < 0.01, and ***P < 0.001 vs vehicle; #P < 0.05 vs. 1 mg/kg sCT; §P < 0.05 vs. 1 mg/kg sCT (Student’s t-test). Data are means ± SE.
and fail to reduce fasting blood glucose. The present studies have introduced a novel oral DACRA, namely KBP-042, which was identified through in vitro receptor pharmacology screening and in vivo testing in animal models of obesity and type 2 diabetes.

The in vitro receptor screening showed that eel calcitonin and sCT induced activation of the receptors to a similar extent (data not shown), and thus sCT was chosen as comparator to KBP-042 due to our previous proof-of-concept studies for oral sCT in DIO and ZDF rats (14, 15). In general, KBP-042 demonstrated a more potent activation of the amylin and calcitonin receptors than sCT, resulting in more pronounced induction of cAMP and β-arrestin signaling. Additionally, KBP-042 did not activate the CGRP receptor, which contrasts native amylin and analogs observed to exert off-target activation of the CGRP receptor (Ref. 33 and data not shown). Activation of CGRP receptors exerts detrimental effects on insulin secretion (43) as well as vasodilatation, which has been linked to migraines (6), and thus the more restricted agonist profile of KBP-042 could be of therapeutic importance, although this awaits further investigation.

The short-term dose response study in DIO rats, a polygenic obese prediabetic animal model (28), confirmed biological activity of oral KBP-042 in vivo and demonstrated dose-dependent reductions in food intake and body weight and improved glucose tolerance, consistent with previous data obtained for oral sCT in DIO rats (15). To explore whether the anorectic and leptogenic actions of oral KBP-042 were sustainable and superior to oral sCT, we performed a longer-term intervention study in DIO rats. Oral KBP-042 induced a sustained reduction in food intake throughout the study period, which was superior to that of oral sCT. Furthermore, the reduction also appeared to exceed that observed in previous studies of both oral sCT (15) and injectable amylin analogs (32). We speculate that this pronounced reduction in food intake could be attributed partly to the rather unique prolonged or almost irreversible binding of sCT and analogs to both amylin receptors located in area postrema/nucleus tractus solitarii (AP/NTS) of the brainstem (19a, 31). This is further support by a blunting of the anorectic effect of sCT when lesions are introduced in AP/NTS (30). Importantly, oral KBP-042 was superior to both oral sCT and pair-feeding in inducing substantial weight loss in DIO rats, which could indicate an additive effect on energy expenditure, as reported for amylin agonism under certain situations (36, 45), although this was not formally investigated. Supporting previous findings for oral sCT (14, 15), we found a concomitant reduction in hyperinsulinemia and hyperleptinemia in conjunction with improved glucose homeostasis, which could indicate a direct alleviation of insulin and leptin resistance to improve metabolic and glycemic control. In support, amylin administration was recently demonstrated to improve the insulin-sensitizing effect of leptin in DIO mice (22) and to enhance the effect of leptin on energy balance and glycemic control in insulin-resistant diabetic mice (26). Hence, accumulating evidence now highlights the importance of peptides with amylin-like properties in the hyperinsulinemic and hyperleptinemic states, and therefore KBP-042 could be of therapeutic importance, although this awaits further investigation.

To further explore whether oral KBP-042 could exert antidiabetic efficacy, as reported for oral sCT (14), we performed a multiple-dose head-to-head study in ZDF rats, a monogenic obese animal model of type 2 diabetes (39). Oral KBP-042 attenuated diabetic hyperglycemia and dose-dependently reduced fasting and nonfasting blood glucose in conjunction with HbA1c levels. Furthermore, oral KBP-042 markedly improved glucose tolerance and insulin action/sensitivity. As observed in DIO rats, superiority of KBP-042 to sCT was demonstrated at
a treatment dose of 1 mg/kg KBP-042, which resembled the efficacy of sCT at the 2 mg/kg dose. As expected from our previous study (14), the antilipemic effect of this class of therapeutic peptides was associated with a prevention of hypoinsulinemia, which indicated preserved pancreatic β-cell function and, importantly, resulted in increased pancreatic insulin content. Furthermore, the pronounced hyperglucagonemia in the ZDF rat was markedly reduced by oral KBP-042, and concomitantly, pancreatic glucagon content was normalized toward levels observed in control rats. Although the effects on pancreas in the ZDF rats appear to be contradictory to those in the DIO rats, these previously published results (18, 20) are related to the highly different phenotypes of the two animal models. The reason for the increase in fasting plasma insulin in the ZDF rats lies within the protection against β-cell loss and is therefore an indirect effect (20). In support of a directly mediated glucagonostatic action, we confirmed that KBP-042 reduced excessive glucagon secretion during arginine exposure in isolated islets, which has been reported previously for amylin at high doses (2), although a centrally mediated effect is most likely also prevalent for amylin-like peptides (40). Irrespectively, the findings suggest that oral KBP-042 preserves pancreatic secretory function by decelerating the α-cell and β-cell stress induced by hyperglycemia and impaired insulin action. During the study of the glucoregulatory mode of action, we found strong evidence toward enhanced insulin action and increased glucose disposal during IVITT, thus introducing an insulin-sensitizing effect, and again KBP-042 was superior to sCT. Although short-term IVITT is directly mediated glucagonostatic action, we confirmed that insulin in the ZDF rats lies within the protection against insulin resistance and type 2 diabetes. Hence, the present studies introduce oral KBP-042 as a novel pharmacological intervention in obesity and type 2 diabetes.

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AUTHOR CONTRIBUTIONS

REFERENCES


