Overexpressed beta cell CART increases insulin secretion in mouse models of insulin resistance and diabetes

Mia Abels, Matteo Riva, Liliya Shcherbina, Ann-Helen Thorén Fischer, Elin Banke, Eva Degerman, Andreas Lindqvist, Nils Wierup *
Lund University Diabetes Centre, Malmö, Sweden

ARTICLE INFO

Keywords:
Type 2 diabetes
T2D
Cocaine- and amphetamine-regulated transcript (CART)
CARTpt
In vivo
INS-1
OGTT
Abdomes:
Aim: to assess the impact of overexpressed CART on insulin secretion in INS-1 832/13 cells.

Abstract

Impaired beta cell function and beta cell death are key features of type 2 diabetes (T2D). Cocaine- and amphetamine-regulated transcript (CART) is necessary for normal islet function in mice. CART increases glucose-stimulated insulin secretion in vivo in mice and in vitro in human islets and CART protects beta cells against glucotoxicity-induced cell death in vitro in rats. Furthermore, beta cell CART is upregulated in T2D patients and in diabetic rodent models as a consequence of hyperglycaemia. The aim of this study was to assess the impact of overexpressed beta cell CART on islet hormone secretion and glucose homeostasis in a transgenic mouse model.

To this end, mice with beta cell-specific overexpression of CART (CARTtg mice) were generated. CARTtg mice challenged by aging, high fat diet feeding or streptozotocin treatment were phenotyped with respect to insulin and glucagon secretion, glucose homeostasis, and beta cell mass. In addition, the impact of adenoviral overexpression of CART on insulin secretion was studied in INS-1 832/13 cells. CARTtg mice had a normal metabolic phenotype under basal conditions. On the other hand, with age CARTtg mice displayed increased insulin secretion and improved glucose elimination, compared with age-matched WT mice. Furthermore, compared with WT controls, CARTtg mice had increased insulin secretion after feeding a high fat diet, as well as lower glucose levels and higher insulin secretion after streptozotocin treatment. Viral overexpression of CART in INS-1 832/13 cells resulted in increased glucose-stimulated insulin secretion.

Together, these results imply that beta cell CART acts to increase insulin secretion when beta cell function is challenged. We propose that the increase in beta cell CART is part of a compensatory mechanisms trying to counteract the hyperglycaemia in T2D.

1. Introduction

Cocaine- and amphetamine-regulated transcript (CART) [1] is a regulatory peptide with important roles in appetite regulation and feeding behaviour [2,3]. In addition, we have previously demonstrated that CART is a potent regulator of islet function and glucose homeostasis [4]. CART is expressed in the central [5], peripheral [6–8] and enteric [9,10] nervous systems, as well as in endocrine cells in e.g. the adrenal medulla [11], pituitary [11], the GI-tract [9], and the thyroid [12].

We have shown that CART is expressed in the pancreatic islets in rodents, pigs and humans, in nerve fibres innervating the islets [12], and/or in endocrine islet cells [4,13–15]. CART increases insulin secretion in a glucose-dependent fashion both in vivo and in vitro in rodents and in vitro in humans [4,14]. Furthermore, Cart KO mice display diminished glucose-stimulated insulin secretion (GSIS) both in vitro and in vivo [16], demonstrating that CART is necessary for maintaining normal insulin secretion. We have also shown that addition of exogenous CART inhibits glucagon secretion in vitro in mouse and human islets and in vivo in mice [4]. Both effects involve altered exocytosis [4] and whereas the exact mechanisms behind the effect on glucagon remain to be established, the effect on insulin has been shown to involve cAMP/PKA-dependent pathways, as well as altered oscillation pattern of Ca^{2+} and in addition improved synchronization of Ca^{2+} oscillations

Abbreviations: AIR, acute insulin release; CART, cocaine- and amphetamine-regulated transcript; CARTtg, mice with beta cell specific overexpression of CART; CD, control diet; GSIS, glucose-stimulated insulin secretion; HFD, high fat diet; KG, glucose elimination constant; STZ, streptozotocin.

* Corresponding author at: Unit of Neuroendocrine Cell Biology, Lund University Diabetes Centre, Department of Clinical Sciences in Malmö, CRC 91:12, Jan Waldenströms gata 35, 214 28, Malmö, Sweden.

E-mail address: nils.wierup@med.lu.se (N. Wierup).

https://doi.org/10.1016/j.peptides.2022.170747
Received 7 October 2021; Received in revised form 14 January 2022; Accepted 14 January 2022
Available online 19 January 2022
0196-9781/© 2022 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
between mouse beta cells [4,14]. Furthermore, CART protects beta cells from glucotoxicity via reduced apoptosis and increased proliferation in clonal INS-1 832/13 beta cells and rat islets. The effect on proliferation was blocked by inhibition of PKA, PKB, and MEK, and CART induced phosphorylation of CREB, IRS, PKB, FoxO1, p44/42 MAPK, and p90RSK [17]. Moreover, an important role of endogenous beta cell CART was established in INS-1 832/13 cells [18]. Thus, siRNA-mediated silencing of CART caused reduced insulin secretion stimulated with an array of secretagogues (glucose alone, cAMP, K+, and the mitochondrial fuel alpha-KIC), and further supporting that CART acts at late events in the stimulus secretion coupling chain, reduced exocytosis of granules from the ready releasable pool was observed. These effects were paralleled by reduced ATP levels, altered gene expression of exocytosis genes, as well as genes crucial for insulin transcription and processing, as well as for glucose sensing [18]. We have also shown that CART is upregulated in alpha and beta cells in islets from T2D patients [4]. In addition, CART is upregulated in beta-, but not alpha cells in several mouse and rat models of diabetes [4,14], likely as a consequence of increased plasma glucose [4].

Taken together, the available data point towards an important role for CART in beta cell function, and during T2D progression. However, it is not known if endogenous overexpressed CART, as evident in T2D patients and animal models, improves beta cell function in vivo. We hypothesize that CART is upregulated in beta cells during T2D development to prevent hyperglycaemia via increased insulin secretion. To test this, transgenic mice (CARTtg) with beta cell specific overexpression of CART were generated.

We find that overexpression of CART in the beta cells causes increased or restored insulin secretion upon challenge by aging, a high fat diet (HFD), or streptozotocin (STZ) treatment. Together with our previous findings of upregulated beta cell CART expression in T2D patients and rodents, our present findings imply that the upregulation of CART may be part of a homeostatic response trying to prevent hyperglycaemia.

2. Methods

2.1. Animals

Mice were kept in a temperature-controlled room (22 °C) on a 12 h light-dark cycle. Food and water were given ad libitum. The study was approved by the Animal Ethics Committee in Malmö/Lund, Sweden.

2.2. Generation of CARTtg mouse

The human CART (hCART) gene was cloned into the previously modified plasmid pBluescript SK II, kindly provided by Prof. Henrik Semb (Lund, Sweden). In short, the promoter of pBluescript SK II vector was replaced with PDX1 promoter. Further, the EGFP gene was cut from pEGFP-N1 vector and subcloned in pBluescript SK II between BgIII and NotI restriction sites. At the 5′ end of the EGFP fragment the AgeI restriction enzyme was located where h-CART was cloned. Primers were designed to remove the hCART stop codon (bold in the primer sequence) and to insert a 7 amino acid-spacer between hCART and EGFP. The primers used for the insertion of AgeI restriction sites were: 5′-GCTCCACCGGTATGGAGAGCTCCCGC-3′ and 5′-GTGACACCGGTTAAAGCACTAAGCACTTCAGGAG-3′. The fragment of interest was sequenced in order to confirm that it did not contain any mutations and that the hCART gene was in frame with EGFP. The construct was next assessed in the NIH-28 β-cell line. Briefly, NIH-28 cells were transiently transfected with increasing amount of the hCART plasmid using lipofectamine LTX. The cells were then incubated for 24 h and 48 h at 37 °C. After 24 h NIH-28 cells were screened using an inverted fluorescence microscope and were found positive for EGFP. After 48 h, Western blot analysis was performed to detect hCART and EGFP expression. Finally, the plasmid was cut with NotI restriction enzyme allowing isolation of the PDX1 promoter/hCART/EGFP fragment, which was injected in the pronuclei of female C57BL/6 mice. The injection was performed by the Transgenic Core Facility at Lund University, Lund. Transgenic male mice were mated with female wild type (WT) C57BL/6 mice to generate transgenic and WT offspring. Mice were genotyped with PCR using primers targeting the EGFP-gene (fwd: GAC GTA AAC GGC CAC AAC TT, rev: AAC TCG TGC TGC TTC ATG TG).

2.3. Tissue processing for immunohistochemistry

Pancreata were dissected out and weighed before they were fixed overnight at 4 °C in Stefanini’s solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M PBS, pH 7.2). The tissue was washed 3 × 24 h at 4 °C, in Tyrode’s solution containing 10% sucrose, and thereafter frozen. Sections (10 μm) were cut and thaw-mounted on slides.

2.4. Immunohistochemistry

Antibodies were diluted in PBS, pH 7.2, containing 0.25% BSA and 0.25% Triton X-100. Sections were incubated with previously characterised primary antibodies [13] against proinsulin (dilution: 1:5000, code: 9003, EuroDiagnostica, Malmö, Sweden) or CART (1:1280, 12/D, Cocalico Corp, Reamstown, PA), overnight at 4 °C in moisture chambers. Sections were washed 2 × 10 min in PBS with 0.25% Triton X-100 and incubated 1 h at RT with secondary antibody coupled to either Texas Red or AMCA (Jackson, West Grove, PA USA). Sections were rinsed as before and mounted in PBS: glycerol (1:1).

Immunofluorescence was examined in an epifluorescence microscope (Olympus BX60, Olympus, Tokyo, Japan). Images were acquired with a digital camera (Nikon DS-2Mv, Nikon, Tokyo, Japan). For beta cell mass quantification, all islets in at least 9 different parts of each pancreas were analysed using NIS-Elements software (NIS-Elements 3.1, Nikon, Tokyo, Japan). Total insulin positive area and section area was measured and calculated. The ratio achieved was multiplied with pancreas weight to determine the beta cell mass.

2.5. Glucose, insulin and arginine tolerance tests

Before tolerance test, mice were fasted 4 h and anesthetized with 25 mg/kg fluanison/0.8 mg/kg fentanyl (Hypnorm, Veta Pharma, Leeds, UK) and 12.5 mg/kg midazolam (Dormicum, PanPharma, La Selle-en-Luittre, France). Basal blood sample was taken before glucose/arginine administration. For oral glucose tolerance test (OGTT) mice were given 75 mg glucose by gavage. For intravenous glucose tolerance test (IVGTT) glucose (1 g/kg) was administered through the tail vein. For insulin tolerance test (ITT) insulin was injected intraperitoneally (i.p) (1 U/kg). For arginine tolerance test (ATT) mice were injected with arginine (250 μg/g) through the tail vein. Blood samples (20 μl) were taken from the retro orbital plexus at indicated time points. Blood samples were put on ice, centrifuged and plasma was separated and stored at −20 °C until analysis.

2.6. Islet isolation and islet insulin secretion

Islets were isolated as previously described [16]. In short, islets were isolated by collagenase digestion and handpicked under a microscope. They were kept overnight at 37 °C in 95% air and 5% CO2 in RPMI 1640 supplemented with 11.1 mM glucose, 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. For experiments, islets were incubated 30 min in HBSS-balanced salt solution (HBSS, mM) (1.14 NaCl, 4.7 KCl, 1.2 KHPO4, 1.16 MgSO4, 20 HEPES, 25.5 NaHCO3, 2.5 CaCl2, 0.1% BSA) containing 2.8 mM glucose. Thereafter, islets were placed three in each well in a 96-well plate containing 200 μl HBSS with indicated glucose levels. The islets were incubated 60 min in incubator; thereafter 100 μl supernatant was saved for analysis of insulin.
2.7. Induction of insulin resistance by high fat diet

Female CARTtg and WT mice were given high fat diet (HFD) with 60 kcal% fat (D12492, Research Diets, NJ 08901 USA) or control diet (CD) with 10 kcal% fat (D12450B, Research Diets) for 8 weeks as previously described [19]. Tissues were collected at the end of study and processed for histology (see above).

2.8. Induction of diabetes by multiple low doses of streptozotocin

Male mice (age 1.5–3 months) were fasted 4 h before injected i.p. with a low dose-streptozotocin (Sigma), 40 mg/kg dissolved in 0.01 M Na-Citrate (pH 4.5) once daily for 5 days as previously described [20, 21]. After last injection, glucose was measured weekly to follow diabetes progression. Glucose was measured in blood samples from tongue vein using ACCU-CHECK Aviva glucometer (Roche). 5 weeks after STZ-treatment, animals were sacrificed, and pancreatic specimens processed for histology.

2.9. Adenovirus-mediated overexpression of CART in INS-1(832/13) cells and insulin secretion assay

INS-1 832/13 cells were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) containing 2 g/l D-glucose, supplemented with 10 % FBS, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol (Sigma Aldrich). For adenoviral overexpression (OE) INS-1832/13 cells were infected with 0.5 MOI of high titer virus stocks (3.8 × 10^10) encoding human CART (CART cDNA kind gift from Dr Tulin Yanik) in 300 μl of growth medium with 2% FBS. After 2 h of infection, 700 μl of complete medium was added and cells were grown at 37 °C. Cells were harvested for mRNA isolation after 48 h (72 h for Western Blot). GSIS was measured 72 h after CART OE. INS-1 832/13 cells were washed twice and incubated for 2 h in 2.8 mM glucose HBSS, followed by 15-min stimulation in HBSS containing 2.8 mM or 16.7 mM glucose.

2.10. Insulin, glucose, glucagon, and protein measurements from in vivo and in vitro experiments

Insulin (1.9 % intraassay variation and 4.9 % interassay variation) and glucagon (5.4 % intraassay variation and 14.7 % interassay variation) were measured with ELISA (Mercodia, Uppsala, Sweden). Glucose was measured using Infinity™ Glucose (Ox) (Thermo Scientific). Protein concentration was analysed using the Bio-Rad protein assay (Bio-Rad, Hercules, California).

2.11. Statistics

Experimental data were analysed using unpaired Student’s t-test or 2-way ANOVA followed by Bonferroni’s test post hoc. Differences with p < 0.05 were considered statistically significant.

3. Results

3.1. Confirmation of CART overexpression in pancreatic islets

CART overexpression in beta cells was confirmed with Western Blot (Fig. 1a) and qPCR (data not shown) in isolated islets from CARTtg mice. CARTtg islets displayed ~20-fold higher expression of biological active CART 55-102 protein compared with WT islets (Fig. 1a). Overexpression was further verified with immunocytochemistry for CART and expression of EGFP in pancreatic sections. Double staining for CART and insulin showed that all beta cells co-expressed CART and EGFP in CARTtg mice (Fig. 1b), whereas CART positive beta cells were few in WT mice, as previously reported [4].

![Fig. 1. Overexpression of CART in beta cells in CARTtg mice.](image-url) Representative Western Blot against CART 55-102 in isolated islets from WT and CARTtg mice with hypothalamus (hypo) as positive control (a). Immunohistochemistry for CART and insulin, as well as eGFP fluorescence in pancreatic sections (b).
3.2. CARTtg mice have increased insulin secretion and improved glucose elimination with increasing age

CARTtg mice had similar body weight as WT mice at all ages (data not shown). At 3–6 months of age CARTtg mice had normal beta cell mass and islet insulin content. When challenged with glucose, arginine or insulin tolerance tests in vivo, CARTtg mice displayed normal insulin and glucagon secretion, as well as glucose elimination (Electronic supplementary material, (ESM) Fig. 1). However, at the age of 12–14 months female CARTtg mice (n = 14) had 2-fold higher GSIS (AIR, CARTtg: 6.2 ± 2.8 vs. WT: 3.3 ± 3.1 ng/mL, p < 0.01, Fig. 2a-c), lower 20-min glucose (CARTtg: 13.8 ± 1.8, WT: 15.8 ± 2.5 mM, p < 0.03, Fig. 2d) and improved glucose elimination (Kg, CARTtg: 2.5 ± 0.8, WT: 1.8 ± 0.8 %/min, p < 0.02) (Fig. 2e) during IVGTT compared with WT mice (n = 14). To test an age-dependent association, female mice at 7, 10, and 12–14 months were subjected to IVGTT, revealing that age was responsible for 10 % of the variation in AIR (two-way ANOVA, p < 0.05, Fig. 2f). CARTtg mice displayed a trend towards increased AIR in mice at the age of 10 months (n = 6, p = 0.1), whereas CARTtg mice aged 7 months had similar AIR as WT mice (Fig. 2f). Although 12–14 months old CARTtg mice displayed increased AIR during IVGTT, beta cell mass (CARTtg: 2.5 ± 1.4 vs. WT: 1.8 ± 0.7 mg, n = 8 + 9, Fig. 2g), islet insulin content (CARTtg: 15.0 ± 2.6 vs. WT: 12.2 ± 3.1 ng/islets, Fig. 2h) and mRNA expression of Ins1 and Ins2 (data not shown) were similar in both genotypes. Furthermore, there were no differences in insulin secretion or glucose levels during OGTT and ITT (ESM Fig. 2) when comparing CARTtg and WT mice.

3.3. CARTtg mice hyper-secrete insulin when challenge with HFD

Since CARTtg mice secreted more insulin as a consequence of increased age, likely to compensate for the ensuing insulin resistance (demonstrated by increased glucose levels in old WT mice during an ITT compared with young WT mice, ESM Fig. 3), we next provoked female mice with HFD, a commonly used model for insulin resistance and pre-diabetes [22]. After 8 weeks mice (age 3–6 months) fed HFD displayed higher insulin secretion (AUCinsulin, HFD: 791 ± 238 vs. ND: 398 ± 87, p < 0.001; Fig. 3c) and higher glucose levels (AUCglucose, HFD: 1592 ± 96 vs. ND: 1501 ± 146, p < 0.01; Fig. 3d) during an OGTT as well as higher glucose values during an ITT (AUCglucose, HFD: 727 ± 59 vs. ND: 667 ± 46, p < 0.001) compared with mice fed ND, demonstrating established insulin resistance induced by the diet. CARTtg mice fed HFD displayed 2.5-fold higher fasting insulin levels (CARTtg: 4.5 ± 3.2 vs. WT: 1.8 ± 0.6 ng/mL, p < 0.01, Fig. 3a), as well as increased insulin secretion during OGTT (AUCinsulin, CARTtg: 908 ± 239 vs. WT: 674 ± 177 ng/mL, p < 0.01Fig. 3b and c), compared with HFD WT mice. Despite increased insulin secretion, HFD CARTtg mice had reduced glucose elimination (Kg, CARTtg: 0.65 ± 0.40 vs. WT: 1.07 ± 0.53 %/min, p = 0.01) during OGTT (Fig. 3d and e). Glucose levels during IVGTT were also slightly higher in HFD CARTtg mice (2-way ANOVA, p = 0.02, ESM Fig. 4) whereas insulin levels were not significantly different compared with HFD WT mice (AIR, WT: 3.7 ± 1.9 vs. CARTtg: 4.3 ± 1.5 ng/mL, p = 0.4). ITT confirmed that HFD CARTtg mice had reduced insulin sensitivity, compared with HFD WT mice (AUCglucose; CARTtg: 754 ± 47 vs. WT: 703 ± 60 mM, p = 0.02, Fig. 3f and g). Thus, our data suggest that CARTtg mice develop
hyperinsulinemia when fed HFD. Potential explanations for this could be increased beta cell mass or increased insulin secretion efficiency. A trend towards increased beta cell mass was seen in HFD CARTtg (CARTtg: 2.8 ± 1.1 vs. WT: 1.7 ± 0.6 mg, p = 0.056, Fig. 3 h). Static incubations of isolated islets revealed no difference in insulin secretion between the two strains on CD (ESM Fig. 1). However, islets from HFD CARTtg mice displayed robustly increased insulin secretion compared with islets from HFD WT mice (CARTtg: 419 ± 63 vs. WT: 180 ± 49 pg/islet/h, p < 0.001, Fig. 3 i).

3.4. CARTtg mice display improved glucose homeostasis after STZ treatment

We next assessed how overexpression of beta cell CART affects beta cell function in a model of hypoinsulinemia. To this end, we studied CARTtg and WT male mice subjected to multiple low-dose streptozocin (STZ) treatment, a commonly used procedure to induce partial beta cell death [21], as previously described [20]. As expected, weekly glucose monitoring confirmed markedly elevated plasma glucose in STZ mice compared with vehicle-treated mice one week after last injection of STZ (Vehicle: 7.9 ± 1.2 vs. STZ: 12.5 ± 3.4 mM, p < 0.0001, Fig. 4 a). Three and four weeks after the last STZ injection CARTtg had significantly lower blood glucose levels compared with WT mice (3 weeks: CARTtg: 17.1 ± 3.5 vs. WT: 21.8 ± 2.6 mM, p < 0.001; 4 weeks: CARTtg: 18.6 ± 4.9 vs. WT: 22.2 ± 3.2 mM, p < 0.001; Fig. 4 a). Four weeks after the last injection of STZ, an IVGTT was performed to examine insulin-secreting capacity. While vehicle-treated mice responded with increased insulin secretion peaking at 1 min, STZ mice were unable to increase insulin secretion in response to the glucose load (Fig. 4 b). However, CARTtg mice had higher insulin levels throughout the test (AUCinsulin, CARTtg: 69.4 ± 15 vs. WT: 48.0 ± 7.6, p < 0.05, Fig. 4 c and d), suggesting improved remaining insulin secretory capacity. Glucose levels peaked at 1 min in both untreated and STZ mice (Fig. 4 e), and, as expected, glucose levels in STZ mice were higher throughout the test. Glucose levels from 1–20 min were, however, lower in STZ CARTtg mice compared with STZ WT mice (AUCglucose 1–20 min, CARTtg 557 ± 66 vs. WT: 642 ± 52, p < 0.05, Fig. 4 f). As expected, beta cell mass was reduced as a consequence of STZ-treatment (regardless of genotype; vehicle: 2.12 ± 0.99 vs. STZ 0.44 ± 0.33 mg, p < 0.001, Fig. 4 g), however, beta cell mass was similar in STZ WT mice and STZ CARTtg mice (Fig. 4 g).

3.5. Viral CART overexpression improves insulin secretion in INS-1(832/13) cells

Having established that overexpression of CART in beta cells in vivo improves insulin secretion we next overexpressed CART in a pure beta cell system to rule out indirect non-beta cell and non-islet effects from e.g. adrenal glands and the CNS. To this end, we used adenoviral vectors to overexpress human CART in INS-1 832/13 beta cells. CART overexpression was verified by qPCR for hCART (data not shown). Western blot confirmed ~15-fold higher expression of biological active CART 55-102 (Fig. 5 a), approximately corresponding to the level of overexpression in CARTtg mice. CART overexpression provoked a 20 % increase of 15 min GSIS (GFP 2.1 ± 0.6 vs CART 2.5 ± 0.7 fold change, p < 0.01, Fig. 5 b), demonstrating direct effects of CART overexpression.
Fig. 4. Improved glucose homeostasis and higher residual insulin secretion in male CARTtg mice after streptozotocin (STZ) treatment.
a: CARTtg mice treated with a multiple low dose STZ protocol had lower glucose levels after 3 and 4 weeks compared with STZ-treated WT mice (n = 20). b-f: IVGTT in STZ- and vehicle-treated CARTtg and WT mice (n = 4–6 in each group). b: Insulin secretion all treatment groups. c: Insulin secretion from STZ-treated mice. d: AUC insulin was higher in STZ-treated CARTtg mice compared with STZ-treated WT mice. e, f: Glucose levels were lower from 1–20 min in STZ CARTtg mice compared with STZ WT mice. g: Quantification of beta cell mass showing decreased beta cell mass in STZ-treated mice compared to vehicle-treated mice (regardless of genotype), but no difference between CARTtg and WT mice. *p < 0.05, ***p < 0.001.

Fig. 5. Increased insulin secretion after adenoviral overexpression of CART in INS-1 (832/13) cells. CART overexpression in INS-1 832/13 cells was verified and quantified by Western blot (n = 3, a). Adenoviral overexpression of CART in INS-1 832/13 cells increased 15 min GSIS (b). GFP adenovirus (GFP) was used as negative control (n = 7). *p < 0.05.
on beta cell function.

4. Discussion

We have previously shown that CART is overexpressed as a consequence of elevated glucose in alpha- and beta cells in T2D patients and in beta cells in rodent models of T2D [4]. Based on the fact that CART increases insulin secretion we hypothesised that CART is upregulated in T2D islets as a compensatory mechanism trying to overcome hyperglycaemia via increasing insulin secretion [4]. To investigate this, we generated mice with beta cell specific overexpression (CARTtg mice).

Our results show that CARTtg mice were normoglycaemic and normoinsulinemic under baseline conditions, but displayed elevated insulin secretion during three different challenges, i.e. aging, HFD and STZ treatment. Depending on the circumstances CARTtg mice displayed either unchanged or deteriorated insulin sensitivity compared with WT littermates. Finally, and excluding non-beta cell effects in CARTtg mice, viral overexpression of CART in INS-1 832/13 cells resulted in increased GSIS.

We observed an age-dependent increase in AIR from 6 months up to 12 months, and at the age of 12 months CARTtg mice had 2-fold increased acute insulin release, which was paralleled by improved glucose elimination. This could not be explained by increased insulin synthesis or beta cell mass. One possible explanation could be that the beta cell insulin secretion capacity was increased as a response to increasing demand for insulin, as aging is associated with development of insulin resistance [23,24]. We have previously shown that CART treatment has both acute and long-term effects on insulin secretion and this could partly be explained by increased insulin exocytosis from isolated mouse islets [4]. Furthermore, as the responses to OGTT were similar in both groups, the increase in insulin secretion does not seem to involve altered incretin action. There is no ready explanation for the lack of difference between the groups during OGTT, but it is not inconceivable that the difference in elevated incretin hormones levels during OGTT overrides the effect of overexpressed CART.

HFD feeding in CARTtg mice resulted in increased insulin secretion in vivo and in isolated islets (tested at 11.1 mM glucose). In addition, CARTtg mice had reduced glucose elimination and were less insulin sensitive compared with WT mice. High plasma levels of insulin may desensitise insulin target tissues, therefore, it is possible that endogenous CART triggers excessive insulin secretion, which in turn leads to reduced insulin sensitivity. This has previously been shown in mice overexpressing extra copies of the insulin gene, which have elevated levels of basal plasma insulin [25], similar to what we observed in CARTtg mice fed HFD. These insulin-overexpressing mice have impaired glucose elimination and decreased insulin sensitivity, and the authors conclude that basal hyperinsulinemia leads to insulin resistance associated with disturbances in glucose metabolism and insulin secretion [25]. Guided by siRNA experiments in INS-1 832/13 cells showing that intracellular CART affects insulin secretion by acting on multiple levels, including an effect on late events of exocytosis, we believe that the effects of CART on insulin secretion in CARTtg mice are intracellular [18]. We have not been able to measure CART in mouse plasma, despite using several different kits from well-known manufacturers. As an alternative strategy we measured circulating eGFP and found that WT mice had no detectable eGFP as expected, whereas CARTtg mice (n = 12) displayed 81 ± 17 pg/mL eGFP at basal levels. Stimulation with arginine i.v. caused increased plasma concentrations after 20 min in CARTtg mice (0 m in. 78 ± 8 vs. 20 m in. 93 ± 11 pg/mL, p = 0.01; n = 7). Although it remains to be proven whether CARTtg mice have elevated plasma levels of CART, one may speculate that secretion of CART from the islets could interfere with insulin signalling in target tissues. This notion is supported by unpublished observations of altered insulin signalling in adipose tissue and liver of CARTtg mice and by previous observations showing that CART alters insulin signalling in rat adipocytes [26].

To study beta cell overexpression of CART in a hypoinsulinemic model, we treated CARTtg mice with a multiple low dose of STZ. This protocol, as opposed to a high single dose that destroys the majority of beta cells, leaves the mice with some residual insulin secretion capacity [21,27]. Notably, CARTtg mice displayed lower glucose levels compared with WT mice at 3 and 4 weeks after STZ treatment. CARTtg mice also displayed lower glucose levels and higher insulin levels during an IVGTT. On the other hand, beta cell mass was similar in both groups after STZ treatment. Even though CART affects apoptosis and proliferation in vitro [17,18] and a trend towards increased beta cell mass was seen in HFD CARTtg mice, our present data does not favour any major role for CART as a regulator of beta cell mass. This notion gains support from our previous observations in Cart KO mice, which had similar islet size and islet number as WT mice [16]. Rather, the main function of CART seems to be to enhance insulin secretion capacity, which agrees with our previous result demonstrating that CART increases GSIS via enhanced beta cell exocytosis and improved synchronisation of Ca2+ oscillation within the islets [4]. This is consistent with the observations that Cart KO mice have diminished GSIS [16] and that silencing of Cart in INS-1 832/13 cells decreases insulin secretion via e.g. reduced exocytosis of insulin granules from the ready releasable pool [18].

We also showed that overexpression of CART in a pure beta cell system resulted in increased insulin secretion, even without challenging the system as was the case in CARTtg mice. Thus, it is unlikely that the increased insulin secretion in CARTtg mice was secondary to non-islet actions of CART e.g. in the central nervous system.

Even though we have previously shown that addition of exogenous CART reduces glucagon secretion [4], CARTtg mice and WT responded similarly to an arginine tolerance test for glucagon secretion capacity in young mice. Whether this is related to adaptations due to life-long overexpression of CART is not known, and it remains to be established whether metabolic challenging by age, STZ or HFD affects the impact of beta cell CART-overexpression on glucagon secretion.

Taken together, our data show that overexpression of CART in beta cells, as seen in patients and diabetes models, causes increased insulin secretion, after intravenous or oral glucose administration, in situations of beta cell stress, induced by e.g. aging, insulin resistance and reduced beta cell mass. Based on the present and previous data we suggest that the upregulation of beta cell CART is a homeostatic response trying to counteract hyperglycaemia.

Contribution statement

All authors contributed to each of the following: (1) substantial contribution to the conception and design, acquisition of data and/or analysis and interpretation of data; (2) drafting the article and/or revising it critically for important intellectual content; and (3) final approval of the version to be published. NW is guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of Competing Interest

The authors declare that there is no duality of interest associated with this manuscript.

Acknowledgements

This study was supported by grants from the Swedish Research Council (Project grants: (2020-01017, 2017-00862, 521-2012-2119, 522-2008-4216, K2009-55X2111-01-4), Linneanum grant to Lund University Diabetes Centre 349-2006-237, Strategic Research Area grant EXODIAB2009-1039), from European Foundation For the Study of Diabetes/Merk Sharp & Dohme, Royal Physiological Society in Lund, Faculty of Medicine at Lund University, Region Skåne, Gyllenstierna Kraruperup, Albert Pålhlsson, and from Novo Nordisk, Crafoord and
Swedish Diabetes Foundation.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.peptides.2022.170747.

References

[1] J. Douglass, A.A. McKinzie, P. Couceyro, PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine, J. Neurosci. 15 (3 Pt 2) (1995) 2471–2481.

[2] P. Kristensen, M.E. Judge, L. Thim, U. Ribel, K.N. Christjansen, B.S. Wulff, J. F. Clausen, P.B. Jensen, O.D. Madsen, N. Vrang, P.J. Larsen, S. Hastrup, Hypothalamic CART is a new anorectic peptide regulated by leptin, Nature 393 (6680) (1998) 72–76.

[3] P.D. Lambert, P.R. Couceyro, K.M. McGirr, S.E. Dall Vechia, Y. Smith, M.J. Kuhar, CART peptides: novel addiction- and feeding-related neuropeptides, Y. Synapse 29 (4) (1998) 293–298.

[4] M. Abels, M. Riva, H. Bennet, E. Ablaqvist, O. Dyachok, V. Nagaraj, L. Scherbhina, R.G. Fred, W. Foon, M. Sorhede-Winzell, J. Fadista, A. Lindqvist, L. Kask, R. Sathanoori, M. Dekker-Nitert, M.J. Kuhar, B. Ahren, C.B. Wollheim, O. Hansson, A. Tengholm, M. Fex, E. Renstrom, L. Group, V. Lyssenko, N. Wierup, CART is overexpressed in human type 2 diabetic islets and inhibits glucagon secretion and increases insulin secretion, Diabetologia (2016).

[5] M.J. Kuhar, S.E. Dall Vechia, CART peptides: novel addiction- and feeding-related neuropeptides, Trends Neurosci. 22 (7) (1999) 316–320.

[6] E.O. Koylu, P.R. Couceyro, P.D. Lambert, M.J. Kuhar, Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain, J. Comp. Neurol. 391 (1) (1998) 115–132.

[7] S.L. Dunn, D.A. Chiana, M.J. Kuhar, R.G. Fred, W. Foon, M. Sorhede-Winzell, J. Fadista, A. Lindqvist, L. Kask, R. Sathanoori, M. Dekker-Nitert, M.J. Kuhar, B. Ahren, Kart, C. B. Wollheim, O. Hansson, A. Tengholm, M. Fex, E. Renstrom, L. Group, V. Lyssenko, N. Wierup, CART is overexpressed in human type 2 diabetic islets and inhibits glucagon secretion and increases insulin secretion, Diabetologia (2016).

[8] E. Ekblad, M. Kuhar, N. Wierup, F. Sundler, Cocaine- and amphetamine-regulated transcript peptide immunoreactivity in the rat spinal preganglionic nuclei, Neurosci. Lett. 294 (3) (2000) 143–146.

[9] C. Broberger, K. Holmberg, M.J. Kuhar, T. Hokfelt, Cocaine- and amphetamine-regulated transcript in the rat vagus nerve: a putative mediator of cholecystokinin-induced satiety, Proc. Natl. Acad. Sci. U. S. A. 96 (23) (1999) 13506–13511.

[10] E. Ekblad, M. Kuhar, N. Wierup, F. Sundler, Cocaine- and amphetamine-regulated transcript: distribution and function in rat gastrointestinal tract, Neurogastroenterol. Motil. 15 (5) (2003) 545–557.

[11] P. Couceyro, M. Paquet, E. Koylu, M.J. Kuhar, Y. Smith, Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity in myenteric plexus neurons of the rat ileum and co-localization with choline acetyltransferase, Synapse 30 (1) (1998) 1–8.

[12] N. Wierup, A. Gunnarsson, T. Ekbald, F. Sundler, Characterisation of CART-containing neurons and cells in the porcine pancreas, gastro-intestinal tract, adrenal and thyroid glands, BMC Neurosci. 8 (2007) 51.

[13] N. Wierup, M. Kuhar, B.O. Nilsson, H. Mulder, E. Ekbald, F. Sundler, Cocaine- and amphetamine-regulated transcript (CART) is expressed in several islet cell types during rat development, J. Histochem. Cytochem. 52 (2) (2004) 169–177.

[14] N. Wierup, M. Bjorkqvist, M.J. Kuhar, H. Mulder, F. Sundler, CART regulates islet hormone secretion and is expressed in the beta-cells of type 2 diabetic rats, Diabetes 55 (2) (2006) 305–311.

[15] N. Wierup, F. Sundler, CART is a novel islet regulatory peptide, Peptides 27 (8) (2006) 2031–2036.

[16] N. Wierup, W.G. Richards, A.W. Bannon, M.J. Kuhar, B. Ahren, F. Sundler, CART knock out mice have impaired insulin secretion and glucose intolerance, altered beta cell morphology and increased body weight, Regul. Pept. 129 (1–3) (2005) 203–211.

[17] R. Sathanoori, B. Olde, D. Erlinge, O. Goransson, N. Wierup, Cocaine- and amphetamine-regulated transcript (CART) protects beta cells against glucotoxicity and increases cell proliferation, J. Biol. Chem. 288 (5) (2013) 3208–3218.

[18] L. Scherbhina, A. Edlund, J.I. Esquerra, M. Abels, Y. Zhou, E. Tottonson-Lakso, C. B. Wollheim, O. Hansson, L. Eliasson, N. Wierup, Endogenous beta-cell CART regulates insulin secretion and transcription of beta-cell genes, Mol. Cell. Endocrinol. (2017).

[19] J. Ahren, B. Ahren, N. Wierup, Increased beta-cell volume in mice fed a high-fat diet: a dynamic study over 12 months, Islets 2 (6) (2010) 353–356.

[20] K.L. Egerod, C. Jin, P.S. Petersen, N. Wierup, F. Sundler, B. Holst, T.W. Schwartz, Beta-cell specific overexpression of GPR39 protects against streptozotocin-induced hyperglycemia, Int. J. Endocrinol. 2011 (2011) 401258.

[21] A.A. Liko, A.A. Rosini, Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus, Science 193 (4251) (1976) 415–417.

[22] R.S. Surwit, C.M. Kuhn, C. Cochrane, J.A. McCubbin, M.N. Feinglos, Diet-induced type II diabetes in C57BL/6J mice, Diabetes 37 (9) (1988) 1163–1167.

[23] R.A. Defronzo, Glucose intolerance and aging: evidence for tissue insensitivity to insulin, Diabetes 28 (12) (1979) 1095–1101.

[24] R.L. Fink, G.G. Kohlerman, J. Griffin, J.M. Olefsky, Mechanisms of insulin resistance in aging, J. Clin. Invest. 71 (6) (1983) 1523–1536.

[25] M.H. Shanik, Y. Xu, J. Skrha, R. Dankner, Y. Zick, J. Roth, Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? Diabetes Care 31 (Suppl 2) (2008) S262–S268.

[26] E. Banke, M. Riva, L. Shcherbina, N. Wierup, E. Degerman, Cocaine- and amphetamine-regulated transcript: distribution and function in rat gastrointestinal tract, J. Histochem. Cytochem. 52 (2) (2004) 169–177.

[27] M. Abels et al.