The role of CART in islet biology

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A R T I C L E   I N F O

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A B S T R A C T

Cocaine- and amphetamine-regulated transcript (CART) is mostly known for its appetite regulating effects in the central nervous system. However, CART is also highly expressed in the peripheral nervous system as well as in certain endocrine cells. Our group has dedicated more than 20 years to understand the role of CART in the pancreatic islets and in this review we summarize what is known to date about CART expression and function in the islets. CART is expressed in both islet cells and nerve fibers innervating the islets. Large species differences are at hand and CART expression is highly dynamic and increased during development, as well as in Type 2 Diabetes and certain endocrine tumors. In the human islets CART is expressed in alpha cells and beta cells and the expression is increased in T2D patients. CART increases insulin secretion, reduces glucagon secretion, and protects against beta cell death by reducing apoptosis and increasing proliferation. It is still not fully understood how CART mediates its effects or which receptors that are involved. Nevertheless, CART is endowed with several properties that are beneficial in a T2D perspective. Many of the described effects of CART resemble those of GLP-1, and interestingly CART has been found to potentiate some of the effects of GLP-1, paving the way for CART-based treatments in combination with GLP-1-based drugs.

1. Introduction

The mRNA for cocaine- and amphetamine-regulated transcript (CART) was discovered in 1995 as a previously uncharacterized mRNA being upregulated in rat striatum after acute administration of cocaine or amphetamine [1]. However much earlier, a fragment of the CART peptide was identified in extracts of sheep hypothalamus [2]. CART has emerged as a classic brain-gut peptide with a wide range of biological effects as a neurotransmitter and as a hormone [3–7]. CART expression has been demonstrated in the central, peripheral and enteric nervous systems [4,8,9], in adipose tissue [10], as well as in endocrine cells in pancreatic islets [11–16], the thyroid [17] and the adrenal medulla [17,18].

In the brain, CART mRNA and protein is highly expressed in the arcuate nucleus (Arc), the lateral hypothalamic area (LHA), the paraventricular nucleus (PVN) and the nucleus accumbens (NAc); brain nuclei involved in the regulation of food intake [3,7,8]. Intracerebroventricular (ICV) administration of CART peptide inhibits food intake in rats, while administration of neutralizing antibodies stimulates food intake [19]. CART mRNA expression in the Arc is decreased in food-deprived animals or in animals with disrupted leptin signaling [8]. Moreover, chronic ICV administration of CART peptide inhibits food intake, while Cart KO mice develop obesity [13,20]. In humans, a nonsense mutation within the NH2-terminal CARTPT region is associated with reduced resting energy expenditure and the mutation co-segregates with obesity [21]. It has also been shown that CARTPT contributes to the genetic risk for obesity in a Caucasian population [22], but hitherto CART genetic variants have not been associated with obesity, T2D risk or related traits in GWAS studies [23–26]. Taken together, there is a body of evidence supporting that CART is an anorexigenic peptide.

In the GI tract CART is widely expressed in the enteric nervous system of rats [4,27], mice [28], pigs [17], sheep [29], guinea pigs [30] and humans [31]. Except from a moderate attenuation of NO mediated relaxation in the colon [4], the function of CART in ENS has remained enigmatic. Interestingly therefore, CART-containing enteric neurons were recently shown to sense microbes and to regulate circulating glucose levels and feeding behavior [28]. Thus, corroborating the concept of peripheral CART as a regulator of food intake shown earlier [32]. CART expression has also been demonstrated in unidentified enterendocrine cells in the abomasum of sheep [29], in rat antral and duodenal gastrin cells [4], as well as in human L- and K-cells [33] as described below. CART is also expressed in the adipose tissue of humans and rats and exerts insulin-like or insulin-antagonistic effects, depending on the surrounding conditions, in rat adipocytes [10].
Following the discovery of CARTPT mRNA, the amino-acid sequence of the protein was predicted, including a 27 amino-acid leader sequence, suggesting secretion through the regulated secretory pathway. Pairs of basic amino acids in the sequence also suggested the likelihood of cleavage processing [9]. The human CARTPT gene spans approximately 2.0 kb and consists of an approximately 340-nucleotide long proximal promoter region, three exons and two introns [34]. In rodents, Carpt mRNA is found in two alternatively spliced forms resulting in production of propeptides of different lengths; proCART 1-89 and proCART 1-102 (the section encoding amino acids 27-39 is spliced out) [1,35,36] (Fig. 1). In contrast, only proCART 1-89 is found in humans. Notably, the regions encoding the active parts of the CART peptides lie downstream of the spliced region and are intact in both propeptides. The importance of the two forms of proCART, and if they are regulated differently, is unknown. However, from the number of cDNA clones identified, one can infer that proCART 1-89 is the more abundantly expressed form [1]. Furthermore, post-translational processing of CART may differ in central and peripheral tissues [37] resulting in several biologically active fragments. Using neuroendocrine cell lines expressing different pro-hormone convertases (PCs) and PC null mice [38], PC2 was shown to play a major role in the generation of CART 55-102/42-89 peptides, while PC1/3 cleavage resulted in the production of the intermediate peptides CART 33-102/10-89. In addition, PC5/6 participates in CART processing sharing specificity with PC2, but not with PC1/3 [39]. CART peptides are highly conserved across species, sharing 91 % nucleotide identity in human and rat coding regions and 98 % nucleotide identity in the rat and mouse coding regions. Importantly, this results in 95 % amino acid identity between the rat and human proteins, with 100 % amino acid identity at the carboxy-termini (exon 3) [34]. This region is found in all CART peptides and contains six cysteine residues forming disulphide bonds that are crucial for the biological activity of CART [37, 40]. The promoter region of the CARTPT gene contains several transcription factor binding sites including, TATA box, STAT, cAMP responsive element (CRE), AP1, SP1, AP2, E-box, and Pit-1 sites [41]. CARTPT mRNA expression is positively regulated by the cAMP/PKA/CREB-dependent pathways [42–45] and CREB binds directly to the CRE-site of the CARTPT promoter [46]. Furthermore, neuron-restrictive silencer element (pNRSE) has been shown to repress CARTPT expression [47].

CART expression is affected by several hormones. Thus in Arc of ob/ob mice CART mRNA levels are reduced, but restored after leptin injections [8]. Interestingly, ob/ob mice and Zucker diabetic fatty (ZDF)-rats that lack the leptin receptor have increased beta cell CART expression [14], suggesting leptin-independent regulation of CART in beta cells. Also acute administration of corticosterone results in different tissues.

While much is known about the CART gene and protein structure, processing and regulation, it is less well known how CART mediates its effects and via which receptor(s). There is evidence for involvement of pertussis-sensitive Gi/o proteins in mediation of the effects of CART. Pertussis toxin was shown to block CART-stimulated ERK activation in neuronal cell lines [53] and inhibition of voltage-gated Ca\(^{2+}\) channels in neurons after CART treatment [54]. Interestingly, PACAP 6-38 was shown to have antagonistic properties and blocked CART-induced ERK activation in PC12 cells [55]. Furthermore, CART-induced effects on reward were found to be PKA/ERK/CREB-dependent, but not CAMP-dependent [56]. In pancreatic beta-cells, CART has been shown to increase CAMP levels in INS-1 (832/13) cells [15] and to potentiate insulin secretion in the cAMP/PKA-dependent manner from INS-1 (832/13) cells and isolated rat islets [14]. This indicates involvement of G\(\alpha\)s rather than G\(\alpha/o\) in the mediation of the effects of CART in beta-cells and proposes the existence of more than one CART receptor. Recently, CART was elegantly shown by Yosten et al. to be a ligand of the thus far orphan receptor GPR160 and CART was found to regulate neuropathic pain via GPR160-dependent ERK/CREB pathways [5,57]. This is potentially a breakthrough that will facilitate the understanding of the actions of CART, or as Mike Kuhar states: Finally a receptor for CART peptide [58]. Whether GPR160 is involved in mediating the effects of CART in islet cells is not known and needs further investigation.

2. CART expression pattern in islet cells

In all species studied so far, CART is under normal conditions expressed in islet cells and/or in nerve fibers innervating the islets. In normal adult rats, as well as in the African ice rat CART expression is confined to delta cells [12,59,60]. However, during rat development CART is also highly expressed in alpha-, beta-, and PP-cells, but not in ghrelin cells [12]. Adult mice express CART in a subpopulation of the beta cells [16], but during development CART is also expressed in a sub-population of the alpha cells [11]. Islet cells in the pancreas of adult pigs are devoid of CART [17], whereas CART expression has been demonstrated in peripheral islet cells of unknown identity in the islets of adult sheep and cattle [61,62]. In adult humans biological active CART 55-102 is expressed in alpha, beta and PP-cells [16,63]. This expression pattern was also confirmed with in situ hybridization. Even though CART protein is clearly expressed in alpha- and beta cells, more recent observations in our unpublished scRNAseq data from pancreatic preparations from twelve organ donors shows higher mRNA expression of CART in PP-cells than in alpha- and beta cells. The expression pattern during human islet development is less well studied. Our unpublished data shows CART expression predominantly in alpha and PP-cells, but also in subpopulations of beta cells, but not in delta cells during mid gestation of human fetal development. The intracellular localization of CART has been studied in DEX rats using transmission electron microscopy. CART immunoreactivity was localized to the secretory granules of both beta-
and delta cells, suggesting that CART can be secreted from these cells [14]. Further supporting this notion, in mice with PDX-1 driven overexpression of CART-eGFP, elevated circulating levels of eGFP was detected 20 min after i.v. injection of arginine (unpublished observations). In summary, significant species differences in the expression pattern of CART is at hand and should be kept in mind when extrapolating data and choosing model systems. Furthermore, CART has a clear developmental regulation in its expression pattern. Whether this is related to growth-regulating properties of CART needs further studies and will be discussed more in detail later in this review.

3. CART expression pattern in pancreatic neurons

In all species studied so far CART is found in neuronal elements in the pancreas and often in nerve fibers in close contact with the islets. In mice and rats CART is found in pancreatic sensory and parasympathetic nerve fibers emanating from intra-pancreatic ganglia [11–13]. Pigs have CART expression exclusively in parasympathetic nerve fibers and some of these innervate the islets [17]. In the adult bovine pancreas CART expression has been demonstrated in intrapancreatic, presumably parasympathetic, ganglia as well as in nerve fibers innervating both exocrine and endocrine components of the gland [61]. Also, in adult sheep CART containing nerve fibers were found to innervate various parts of the pancreas including the islet perimeter and CART expression was seen in intrapancreatic ganglia [62]. In humans CART expression was reported in intra-pancreatic ganglia and in large nerve trunks, as well as in fibers innervating acini [63]. Our unpublished data shows prominent CART expression in intrapancreatic VIP-containing ganglia as well as in nerve fibers, but not in sensory or adrenergic fibers. The function of CART in pancreatic nerve fibers is not well studied. In the rat CART has been shown to stimulate amylase secretion from the exocrine pancreas via CCK- and acetylcholine-sensitive pathways [64]. Moreover, insulin induces depolarization and increases Ca²⁺ in CART-containing neurons from the nodose ganglion projecting to the pancreas [65]. Interestingly, insulin preferentially targeted CART-containing neurons. These data suggest that CART-containing nerve fibers could be involved in fine tuning of islet hormone secretion and adjusting hormone secretion, and potentially also exocrine secretion to metabolic state. Parasympathetic(cholinergic) nerve fibers are known to stimulate insulin secretion, sensory fibers are known to cause tonic inhibition on insulin secretion [66], whereas adrenergic fibers inhibits insulin secretion [67]. Thus, the expression pattern of CART in neuronal elements of the pancreas suggests that CART could be involved in modulating both stimulatory and inhibitory neurons. It is not yet known whether CART act as a neurotransmitter in these fibers, but observations of CART expression in nerve terminals points in this direction.

4. CART in T2D and regulation of beta cell CART

Despite the large species differences in expression pattern of CART in islet cells, a common pattern appears during T2D development. CART expression is robustly elevated in beta cells of T2D patients and in virtually all (~10) rodent T2D models and knockout mouse strains with perturbed glucose control studied so far in our lab. These include Ob/- Ob, high fat diet fed (HFD) and Hnf1a KO mice, which all have massively increased numbers of CART-containing beta cells, compared to the control strains [16]. Furthermore, beta cell CART expression is even more prominent in the rat T2D models: GK-rats, ZDF-rats [19] and dexamethasone (DEX)-treated rats. Using DEX-treated rats, it has also been shown that beta cell CART expression is highly dynamic, and the upregulation of beta cell CART reverted to normal levels after termination of DEX treatment when glucose levels are again normalized. In addition, the DEX-induced upregulation of CART was prevented by glucose-normalizing insulin treatment [16] or with treatment with glibenclamide (unpublished observations). In islets of human T2D patients, CART expression is, in addition to beta cells, also robustly elevated in alpha cells compared with normoglycemic controls [16]. On the other hand, in rat models of T2D the impact of T2D is specific to beta cells and CART expression in delta cells is unaffected by T2D [14,16].

The mechanism behind increased beta cell CART expression in vivo is not fully understood. It is known from in vitro experiments that CART expression is regulated by glucose in INS-1 cells [15,52] in a bell-shaped manner, reaching peak levels around 11 mM glucose and dropping at toxic levels of glucose [15]. Furthermore, CART expression in isolated rat islets clearly increased after culture in increasing concentrations of glucose [16,68]. These observations together with data showing that beta cell CART expression is reverted or prevented when glucose levels are normalized [16] suggest that elevated plasma glucose levels are driving the elevated beta cell CART expression.

5. The effect of CART on insulin secretion

The effect of CART on insulin secretion has been studied using addition of exogenous CART, by targeting endogenous CART using siRNA and CART KO mice, and by beta cell specific overexpression of CART. In the majority of the experimental settings, CART stimulates insulin secretion in a glucose-dependent fashion. Thus, addition of exogenous CART stimulates GSIS in human (from donors with T2D, as well as normoglycemic donors) and mouse islets, as well as in vivo in mice during an IVGTT [16]. In rat islets and INS-1 832/13 cells CART does not increase insulin secretion when glucose is the only stimuli for insulin release [14,69,70]. On the other hand, CART enhanced cAMP-stimulated GSIS in INS-1 cells, rat islets, mouse islets as well as in vivo in mice during an IVGTT. Also, CART caused an additional increase to that of GLP-1. This additional increase was lost when blocking PKA with H89. CART was found to increase cAMP [15,14], suggesting that CART acts via cAMP/PKA-dependent pathways. Interestingly, CART was found to improve synchronization of Ca²⁺ oscillations between the beta cells within an islet. Even though the exact mechanisms behind this effect of CART remain unknown, synchronisation of beta cells is critical for normal insulin secretion kinetics [71]. CART also changed the oscillation pattern from fast to slow waves; a pattern that is associated with pulsatile insulin secretion [16]. Whole body Cart KO mice have blunted first phase insulin secretion and perturbed glucose elimination [13]. Furthermore, Cart KO islets secrete less insulin in response to glucose and display perturbed expression of PDX-1 and GLUT2. Beta cell specific Cart KOts are not available but experiments with siRNA silencing (KD) of Cart in INS-1 832/13 cells have provided important information about the role of endogenous beta cell CART. Cart KD causes reduced ATP-levels and reduced insulin secretion stimulated with an array of different secretagogues (glucose alone, cAMP, K⁺, and the mitochondrial fuel alpha-KIC), speaking in favor of CART acting at late events in the stimulus secretion coupling. This was tested in patch clamp experiments and Cart KD caused reduced exocytosis of insulin granules from the ready releasable pool. Cart KD also altered expression of genes with key roles in exocytosis as well as insulin processing, glucose sensing and K⁺ control [72]. In agreement, with the INS-1 Cart KD data, addition of exogenous CART increases exocytosis in mouse beta cells [16]. Exocytosis, as well as recruitment of granules to the plasma membrane is promoted by cAMP [73], and it is not inconceivable that the effect of CART on exocytosis is mediated via cAMP/PKA-dependent mechanisms. Moreover, Cart KD in INS-1 cells also caused lowered insulin production, likely as a consequence of lower expression of several key beta cell regulatory transcription factors.

A perhaps overlooked, but potentially important, control mechanism for meeting the metabolic demands for hormone secretion is regulation of islet blood flow [74]. In rats CART caused an inhibition of islet blood flow and reduced the glucose-induced elevation of islet blood flow, without affecting blood flow of the whole pancreas [69], suggesting that CART could be involved in regulation of islet nutrient sensing.

After observing that CART is upregulated in T2D beta cells we aimed to test whether CART is upregulated in T2D islets as a compensatory...
mechanism trying to overcome hyperglycaemia via increasing insulin secretion. To this end, two mouse lines with PDX1-driven overexpression of Cart were constructed. CARTtg mice were normoglycaemic and normoinsulinaemic under normal conditions, but displayed elevated insulin secretion during three different challenges, namely aging, HFD and streptozotocin (STZ) treatment. This could in part be explained by increased beta cell mass and increased insulin secretion capacity. In aged mice and upon STZ treatment, CARTtg mice had improved glycemia compared with wt control mice. Finally, and excluding non-beta cell effects in CARTtg mice, viral overexpression of Cart in beta cells, as seen in human, rat and mouse islets, as well as in vitro, positively regulated by glucose, the available data suggest that the upregulation of beta cell CART in T2D is a homeostatic response trying to counteract hyperglycaemia.

6. CART inhibits glucagon secretion

The available literature shows that CART reduces glucagon secretion in vitro in human, rat and mouse islets, as well as in vivo in mice [14,16]. In human islets the effect was glucose dependent and only observed at stimulatory glucose concentrations. In mouse islets addition of CART blunted alpha cell exocytosis in patch clamp experiments. The mechanistic basis for this seemingly direct inhibitory effect of CART on alpha cell glucagon secretion needs further investigation. However, it should be noted that only a few hormones have direct inhibitory effects on glucagon secretion, e.g. GLP-1 and somatostatin [75,76]. It should be mentioned that in a glucagon-producing hamster cell line (InR1G9) CART (from an undisclosed source) did not significantly inhibit glucagon secretion [77]. CART expression is increased in human T2D alpha cells, but the mechanisms behind this upregulation or the functional consequences are not fully understood. One plausible explanation could be that CART acts to suppress exaggerated glucagon secretion as evident in T2D [78].

In a recent study shRNA silencing of Ins1 in αTC6 cells resulted in lower glucagon mRNA and protein, as well as perturbed Ca²⁺ oscillations in response to glucose and insulin. Interestingly, these effects were paralleled by blunted CART expression [79]. Whether the reduction in CART is directly involved in, is a consequence of the perturbed alpha cell function or is a direct effect of Ins1 KD remains to be established. Nevertheless, the data is intriguing in view of the inhibitory effects of CART on glucagon secretion and a role for CART as a facilitator of intra-islet signalling between beta- and alpha cells is not inconceivable.

7. CART regulates beta cell growth

Observations that CART expression is increased in different situations of growth, e.g. fetal development, beta cell mass expansion in T2D models, and in islet cell tumors [80] formed the rationale for studies aimed to investigate if CART can act as a regulator of cell growth. Using INS-1 832/13 cells and rat islets CART was found to exert protective effects against glucotoxicity-induced cell death. The effect size was similar to that of 10 nM GLP-1, but in contrast to the effect of CART on insulin, there was no additive effect of CART on GLP-1-mediated cell protection. CART also increased proliferation in INS-1 832/13 cells, an effect that was blocked by PKA, PKB, and MEK1 inhibitors. In addition, CART induced phosphorylation of CREB, IRS, PKB, FoxO1, p44/42 MAPK, and p90RSK, all key mediators of cell survival and proliferation [15]. Furthermore, CART siRNA caused increased apoptosis (caspase 3/7 activity) in INS-1 832/13 cells, without affecting general cell viability [72]. On the other hand, in vivo data in Cart KO mice and CARTtg mice does not favor a major role for CART in regulating beta cell mass. Cart KO mice have normal islet size and density of islets per pancreas area [13], and even though CARTtg mice on HFD trended towards higher beta cell mass, CARTtg mice had similar beta cell mass as wt controls after STZ treatment or after ageing. It is not inconceivable that the divergent data are explained by redundant mechanisms in the mouse models, and inducible beta cell specific Cart KOs are warranted to address the role of CART as a regulator of beta cell growth in vivo.

8. CART is a regulator of incretin hormone secretion and production

Guided by findings in animal studies [4,29], CART expression in the human GI-tract was studied. CART was found to be expressed in subpopulations of GIP-expressing K-cells, as well as GLP-1 expressing L-cells [33]. Furthermore, siRNA experiments in STC1 cells and GLUTag cells showed that CART is a stimulator of GLP-1 secretion and mRNA and protein expression. The effect of Cart siRNA on GIP expression was less prominent. However, i.v. administration of CART during an OGTT in mice resulted in elevated circulating levels of both GIP and GLP-1. In humans, CART was released in response to a mixed meal test, but not
after an oral glucose load. This is line with CART being regulated by fatty acids, but not glucose in STC1 cells and GLUT4 cells [33]. Even though the exact cellular source of this elevated circulating CART is not known, the data clearly show that CART is a regulator of incretin hormone secretion and production and a role for CART in these processes during healthy and T2D conditions should be further investigated.

9. Concluding remarks

CART expression in the islets is highly dynamic and there are significant species differences in expression pattern that need to be considered when extrapolating data and choosing model systems. Since the discovery of CART in rat delta cells more than two decades ago pieces have been progressively added to the puzzle to understand the function of CART in islets cells. Clearly, CART stimulates insulin secretion, inhibits glucagon secretion and protects against glucotoxicity-induced beta cell death. Although it remains to be established via which receptor CART acts in islet cells, the mechanisms behind the effect on insulin secretion and cell growth are now fairly well understood. On the other hand, further studies are needed to elucidate the mechanisms behind the effect on glucagon secretion. Observations showing that CART is upregulated in alpha- and beta cells in T2D patients and in beta cells of T2D rodent models as consequence of elevated plasma glucose, suggest that the upregulation of CART in T2D is a homeostatic response trying to counteract hyperglycaemia via increased insulin secretion and reduced glucagon secretion. This together with observations that CART increases GLP-1 and GIP secretion, GLP-1-stimulated insulin secretion, insulin secretion in islets from T2D donors, point at CART-based substances as a therapeutic avenue for T2D (Fig. 3).

10. Future perspectives

Although multiple hypothesis-driven studies have provided important information on the role of CART in islets cells, holistic studies are lacking and could potentially shed light on overlooked mechanisms behind the effects of CART. Furthermore, it remains to be established which receptor that mediates the effects of CART in islets cells. In view of the potentially glucose lowering effects of CART, the effect of long-term CART administration in a T2D model should be evaluated.

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Data availability

Data will be made available on request. No data was used for the research described in the article.

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