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Cocaine- and Amphetamine-Regulated Transcript is Expressed in Adipocytes and Regulate Lipid- and Glucose Homeostasis

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Abstract

Cocaine- and amphetamine-regulated transcript (CART) is a regulatory peptide expressed in the nervous system and in endocrine cells, e.g. in pancreatic islets. CART deficient mice exhibit islet dysfunction, impaired insulin secretion and increased body weight. A mutation in the CART gene in humans is associated with reduced metabolic rate, obesity and diabetes. Furthermore, CART is upregulated in islets of type-2 diabetic rats and regulates islet hormone secretion *in vitro*. While the function of CART in the nervous system has been extensively studied, there is no information on its expression or function in white adipose tissue. CART mRNA and protein were found to be expressed in both subcutaneous and visceral white adipose tissue from rat and man. Stimulating rat primary adipocytes with CART significantly potentiated isoprenaline-induced lipolysis, and hormone sensitive lipase activation (phosphorylation of Ser 563). On the other hand, CART significantly potentiated the inhibitory effect of insulin on isoprenaline-induced lipolysis. CART inhibited insulin-induced glucose uptake, which was associated with inhibition of PKB phosphorylation.

In conclusion, CART is a novel constituent of human and rat adipocytes and affects several biological processes central in both lipid- and glucose homeostasis. Depending on the surrounding conditions, the effects of CART are insulin-like or insulin-antagonistic.

Abbreviations: CART-cocaine- and amphetamine-regulated transcript, HSL-hormone sensitive lipase, PDE3B-phosphodiesterase-3B, PKA-protein kinase A, PKB-protein kinase B, T2D-type 2 diabetes, WAT-white adipose tissue

Keywords

adipocytes; cocaine- and amphetamine-regulated transcript; cellular signalling; lipid metabolism; glucose metabolism

1. Introduction

Obesity and type 2 diabetes (T2D) are increasing in a rapid pace in the western world [1]. Adipose tissue is central in maintaining lipid and glucose homeostasis and an expanding adipose tissue leads to increased free fatty acid (FFA) release from the adipocytes, altered adipokine release and low-grade inflammation [2].

Cocaine- and amphetamine-regulated transcript (CART) is an anorexigenic peptide [3,4,5,6] highly expressed in the brain, the peripheral nervous system [7,8,9] as well as in the enteric nervous system [10,11]. CART is also expressed in pancreatic islet cells and in nerve fibers innervating the islets in rats, mice, pigs [10,12,13,14] and humans (Riva *et al,* unpublished data). In addition, CART is expressed in neuroendocrine tumours in rats [14] and humans [15,16,17]. Furthermore, CART is expressed in endocrine cells in the pituitary, adrenal medulla and thyroid [8,18]. CART can also be found in the circulation [19]. The receptor for CART has not yet been identified [3].

In addition to the inhibitory action on appetite, CART is also an important regulator of islet hormone secretion and necessary for normal islet function. CART -/- mice exhibit impaired glucose tolerance due to islet dysfunction. Furthermore, CART -/- mice have increased body weight without an increase in food intake [12,20]. On the other hand, CART is highly upregulated in the β-cells of two different T2D rat models [21]. Interestingly, mutations in the human CART gene has been linked to obesity, reduced metabolic rate and elevated incidence of T2D [22,23,24]. Although the importance of CART in obesity is well established, it is not known whether CART plays a role in regulation of adipocyte function. We aimed to investigate CART expression in human and rat white adipose tissue (WAT) and potential effects of CART on cellular signaling and on lipid- and glucose metabolism in rat adipocytes. We show that CART is expressed in both human and rat adipocytes where it affects several biological processes central in lipid- and glucose homeostasis.

2. Materials and methods

2.1 Ethics statement

Animal experiments were approved by the Animal Ethics Committee in Lund and Malmö, Sweden (ethical permit numbers M120-10 and M212-09) and were carried out in accordance with EU directives for animal experiments. Animals were kept under standardized conditions in the animal house facilities and all efforts were made to minimize suffering. Human omental and subcutaneous WAT was obtained from obese patients undergoing gastric bypass operations or from non-obese patients undergoing cholecystectomy operations. Written consent was given and ethical approval was obtained from the Human Ethics Committee in Lund, Sweden. All work was carried out in accordance with the Declaration of Helsinki.

2.2 Materials

For adipocyte stimulation isoprenaline (SIGMA), insulin (Novo Nordisk A/S, Målöv, Denmark) and CART 55-102 (kind gift from Dr. Lars Thim, Novo Nordisk A/S) were used. All primary antibodies used in western blotting, [anti-HSL(pS563) and anti-Akt(pS473)] were from Cell Signaling Technologies (Boston, USA) except for the anti-GAPDH which was from SIGMA (St. Louis, USA). The secondary anti-mouse and anti-rabbit antibodies were from GE Healthcare (Buckinghamshire, UK) and Thermo Scientific (Rockford, USA) respectively.

2.3 Isolation of primary rat adipocytes

Primary adipocytes were isolated from epididymal fat pads from male Sprague-Dawley rats (age: 35-43 days, B&K Universal, Stockholm, Sweden) by collagenase digestion [25]. The cells were diluted in Krebs Ringer-HEPES (KRH) buffer, pH 7.5 with 1% bovine serum albumin (BSA), 2 mM glucose and 200 nM adenosine, to 1.5%-30% suspensions depending on the following method.

2.4 RT-PCR and QPCR

Subcutaneous and epididymal adipose tissue from rat, as well as omental and subcutaneous WAT from obese patients undergoing gastric bypass operations or from non-obese patients undergoing cholecystectomy operations were treated with QIAzol (Qiagen, Stanford, USA) and homogenized using 20 strokes of glass/Teflon homogenizer. Total RNA was isolated using the Qiagen RNeasy Lipid Tissue mini-kit (Qiagen) according to the manufacturer's instructions and DNase treatment was performed with DNaseI (Ambion, Austen, USA). Retrotranscription of 1 μg of RNA into cDNA was carried out using Precision^{TM} reverse transcription kit (Primer Design Ltd, UK). 50 ng of each cDNA sample were used for PCR reaction as follow: 10 min at 95°C, followed by 1 min at 95°C, 1 min at 50°C, 1 min at 72°C for 50 cycles. The products were loaded on a 1% agarose gel. Bands were cut and DNA was extracted using GeneJET^{TM} gel extraction kit (Fermentas GmbH, Germany) and sent for sequencing to DNA Technology (Risskov, Denmark). The primer sequences used were CART-sense: -5'-TTAACAACAATAAAGTTTGCGTTCC-3', CART-antisense: 5'-CACACATACCAACACCATTCAAG-3', designed to be specific for both rat and human. cDNA from INS-1 (832/13) cells was used as positive control and PCR mixture without template as negative control. Quantitative real-time polymerase chain reaction (QPCR) for CART and two endogenous controls (Polr2a and Hprt) was performed with 50 ng cDNA and TaqMan Expression PCR Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). Expression levels were calculated using the ΔΔCt method. TaqMan expression assays used for rat were: CART (Rn01645174_m1), Hprt1 (Rn01527840_m1), Polr2a (Rn01752026_m1); for human: CART (CARThuman T4ca), HPRT1 (4326321E), PolR2a (Hs00172187_m1) (Applied Biosystems).

2.5 Immunocytochemistry

Human subcutaneous WAT from obese patients undergoing gastric bypass operation as well as visceral WAT from female Sprague-Dawley rats (age: 3 months, n=3) was used. Specimens were fixed and stained for CART using previously characterized [13] CART antibodies (Kind gift from Dr. Michael J Kuhar, Emory University, Atlanta, USA) as previously described [13]. The specificity of immunostaining for CART was tested using primary antisera pre-absorbed with excess amount of homologous antigen (100 μg of peptide per ml antiserum in working dilution).

The specificity of the CART antiserum was further verified by lack of staining in intestinal specimens of CART -/- mice [12].

2.6 Lipolysis

Lipolysis was measured by incubating 5% cell suspension at 37°C for 30 min in duplicates with stimuli as indicated. Stimulations were stopped on ice. The medium was removed from the floating adipocytes and transferred to new tubes for enzymatic measurement of glycerol release with the method of Dole and Meinertz [26].

2.7 Western Blotting

10% cell suspensions were incubated at 37°C for 10 min with stimuli as indicated. Stimulations were stopped by washing with BSA free KRH buffer and cells were lysed in a buffer containing 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 0.05 mM Na-orthovanadate, 50 mM NaF, 5 mM Na-pyrophosphate, 0.27 M sucrose, 1 mM dithioerythriol, 1 mM PMSF, 10 mg/ml leupeptin, 1 mg/ml pepstatin, 10 mg/ml antipain and 1% NP40, pH 7.4. Samples were centrifuged at 13 000 x g for 10 min at 4°C, supernatant transferred to new tubes and protein concentrations determined using the Bradford method [27]. Total cell lysates (20 μg protein) were mixed with sample buffer and subjected to electrophoresis on 7.5% bisacrylamide gels. Proteins were transferred to Hybond-C Extra membranes (Amersham Biosciences, Uppsala, Sweden), blocked with 10% milk in tris-buffered saline tween-20 (TBST: 50 mM Tris, 150 mM NaCl and 0.1% Tween-20, pH 7.6) for 1 h and subsequently incubated with primary antibodies at 4°C overnight. After washing, all membranes were incubated with secondary antibody conjugated with horse radish peroxidase for 1 h in room temperature and after washing incubated with SuperSignal West Pico ECL (enzymatic chemoluminescence) reagent (Thermo Scientific) for 10 min in room temperature followed by imaging (IR LAS-1000 ECL camera) and quantification(Image Gauge software, Fujifilm, Tokyo, Japan).

2.8 PDE assay

10% cell suspensions were incubated at 37°C for 10 min with stimuli as indicated in triplicates. Stimulations were stopped by removing media and homogenizing the cells in a buffer containing 50 mM TES, 250 mM sucrose, 1 mM EDTA, 0.1 mM EGTA, 10 μg/ml antipain, 10 μg/ml leupeptin and 1 μg/ml pepstatin A, pH 7.4. Homogenates were centrifuged at 5000 x g for 5 min at 4°C and after removal of the fat cake, samples were centrifuged at 35 000 x g for 45 min at

4°C followed by homogenization of the membrane containing pellet. PDE activity was measured as previously described [28].

2.9 Glucose uptake assay

Incorporation of 2-deoxy-D-[1-^{3}H]-glucose was measured as previously described [29] in 30% cell suspensions in Krebs-Ringer bicarbonate-HEPES buffer (120 mM NaCl, 4 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, 0.75 mM CaCl$_2$, 10 mM NaHCO$_3$, 30 mM HEPES, pH 7.4) with 1% BSA, and stimuli as indicated.

2.10 Lipogenesis assay

Incorporation of 3-^{3}H-glucose into cellular lipids was measured as previously described [30], in 1.5% cell suspensions in KRH buffer with 3.5% BSA, 200 nM adenosine, 0.55 mM glucose and stimuli as indicated.

2.11 Statistics

Parametrical Student´s t-test was used to calculate statistical significance. Differences with a p-value ≤ 0.05 were considered significant. Data are presented as means ± SEM.

3. Results

3.1 CART is expressed in rat and human subcutaneous and visceral adipose tissue

RT-PCR revealed that CART mRNA expression was evident in rat subcutaneous and visceral adipose tissue as well as in human subcutaneous and visceral adipose tissue from lean and obese, male and female patients (figure 1A). Sequencing confirmed that the PCR product was the correct and deduced sequence of CART. CART mRNA expression in human and rat WAT was further confirmed with QPCR (data not shown). In accordance, immunocytochemistry for CART peptide revealed robust immunoreactivity in the cytoplasm of human and rat adipocytes (figure 1B). Importantly, preabsorption with CART 55-102 peptide blocked all staining (figure 1B, panel F).

3.2 CART potentiates isoprenaline-induced lipolysis in primary rat adipocytes and potentiates isoprenaline-induced activation of hormone sensitive lipase

Regulation of lipolysis is an important process for maintenance of lipid- and glucose homeostasis [2]. The beta adrenergic agonist isoprenaline provokes increased lipolysis via elevated cAMP

and subsequent phosphorylation of HSL at the activity controlling site Ser 563 [31]. To assess whether CART affects lipolysis, glycerol release from primary rat adipocytes was measured after addition of CART in three different concentrations (1, 10 and 100 nM for 30 min). The dose-response experiments showed effects on isoprenaline-induced lipolysis already at 1 nM. However, since previous studies in pancreatic islets showed that 100 nM CART was maximally effective on insulin secretion [21], this concentration was used subsequently. CART potentiated isoprenaline-induced lipolysis (figure 2A, CART 1 nM: n=5, p=0.049, 10 nM: n=5, p=0.052, 100 nM: n=10, p=0.015) and the effect was paralleled by a potentiation of isoprenaline-induced phosphorylation/activation of HSL (figure 2B, n=8, p=0.003). In the absence of isoprenaline, CART had no effect on lipolysis, however, CART provoked a raise in HSL phosphorylation/activation by itself (figure 2B, n=8, p=0.035).

3.3 CART potentiates insulin-induced inhibition of lipolysis in primary rat adipocytes and activates phosphodiesterase 3B

Addition of CART potentiated the inhibitory effect of insulin on isoprenaline-induced lipolysis (figure 3A, n=5, p=0.038 and p=0.043). Insulin-induced inhibition of lipolysis is mainly mediated via PKB-mediated phosphorylation and activation of the cAMP hydrolyzing enzyme PDE3B [32]. The anti-lipolytic effect of CART was paralleled by increased PDE3B activity (figure 3B, n=5, p=0.033) to the same extent as insulin (figure 3B, n=5, p=0.05).

3.4 CART inhibits insulin-induced glucose uptake, protein kinase B activation and lipogenesis in primary rat adipocytes

In adipocytes insulin stimulates glucose uptake via mechanisms involving activation of PKB and GLUT4 translocation to the plasma membrane [33]. CART reduced insulin-induced glucose uptake (figure 4A, n=5, p=0.026) and the effect was associated with inhibition of insulin-induced phosphorylation of PKB at Ser 473, an activity-controlling site (figure 4B, n=6, p=0.005). Phosphorylation/activation of PKB is also involved in increasing lipogenesis [34]. Accordingly, CART inhibited insulin-induced lipogenesis (figure 4C, n=4, Ins p=0.0013, CART p=0.028).

4. Discussion

Here we show that CART is a novel constituent of human and rat WAT and that the peptide affects several biological processes central in both lipid- and glucose homeostasis. Depending on the surrounding conditions, the effects of CART are insulin-like or insulin-antagonistic.

CART expression in adipose tissue has hitherto only been observed in fish [35]. Here we found that both CART mRNA and protein is expressed in both visceral and subcutaneous adipose tissue from rat and human. In humans, expression of CART was evident in WAT from lean and obese, as well as in male and female patients. The number of observations for humans was limited and further investigations are needed to understand if adipose tissue CART expression is influenced by obesity. Whether CART from adipocytes have any impact on circulating levels of CART (estimated to be about 80 pmol/l in healthy human subjects) [19] or if CART exerts paracrine effects on neighboring cells needs further investigation.

Stimulating adipocytes with beta adrenergic agonists leads to increased intracellular cAMP levels, activation of PKA and subsequent phosphorylation/activation of HSL. This signaling pathway is the main regulator of lipolysis [31]. Our finding that CART potentiates isoprenaline-induced lipolysis in rat adipocytes and potentiates isoprenaline-induced phosphorylation/activation of HSL is in line with previous findings in β-cells where CART potentiates cAMP-enhanced glucose-stimulated insulin secretion via PKA-dependent mechanisms [21]. CART alone caused a small increase in phosphorylation/activation of HSL without an increase in lipolysis. Suggestively, CART does not activate all components required for lipolysis to occur such as perilipin [31].

In the presence of insulin, CART potentiates the ability of insulin to inhibit isoprenaline-induced lipolysis. The anti-lipolytic effect of CART in the presence of insulin could be explained by CART-mediated phosphorylation/activation of the cAMP-hydrolyzing enzyme PDE3B. Insulin-induced phosphorylation and activation of PDE3B has been shown to be the major mechanism whereby insulin antagonizes lipolysis [32]. We found that CART induces activation of PDE3B to the same extent as insulin. PKB has been shown to have a major role in insulin-induced phosphorylation and activation of PDE3B [32]. However, our finding that CART inhibits the insulin-mediated activation of PKB indicates that CART might mediate phosphorylation/activation of PDE3B via other kinases than PKB. PDE3B has several different sites that can be phosphorylated in response to insulin as well as in response to isoprenaline, forskolin and phosphatase inhibitors [36]. It has been suggested that cAMP-mediated activation of PDE3B is an important feedback regulation of cAMP levels [32]. It is not likely that CART utilizes the same cAMP/PKA pool as isoprenaline to induce PDE3B phosphorylation/activation

and feed-back regulation of cAMP since CART alone has no effect on lipolysis. Exactly which kinase(s) is involved in CART mediated regulation of PDE3B needs further investigation. CART-mediated inhibition of insulin-induced activation of PKB as shown in this study is in agreement with inhibition of glucose uptake as well as the inhibition of lipogenesis under the same conditions. In another study, Perwitz *et al* found that brown adipocytes pretreated with CART showed a 25% enhanced insulin induced glucose uptake although without an increase in insulin stimulated PKB phosphorylation/activation. The divergent data suggest that CART has different effects in immortalized cultured brown adipocytes than in primary rat white adipocytes [37].

Other examples of peptides that can induce both insulin-like and insulin-antagonistic effects on white adipocytes are pituitary adenylate cyclase-activating polypeptide (PACAP) [38] and the incretin glucose-dependent insulinotropic polypeptide (GIP) [39]. PACAP and GIP stimulate lipolysis, however, in the presence of insulin both peptides potentiate insulin-induced lipogenesis [38,39].

In summary, regarding the role of CART in adipocyte function, CART regulates several signaling events important in both lipid- and glucose metabolism. However, our data show that the effects of CART on adipocytes are complex. Exactly which effects CART exerts in a specific situation seems to depend upon the hormonal context, e.g. whether cAMP-pathways are activated and/or whether insulin levels are raised. When it comes to regulation of lipolysis, in the fasting situation, CART could have a beneficial effect in potentiating lipolysis to provide energy substrates for the rest of the body, whereas in the fed situation, when insulin levels are increased, CART rather potentiates insulin-induced inhibition of lipolysis. The ability of CART to inhibit insulin-mediated glucose uptake and subsequent lipogenesis could also be physiologically relevant. In the fed situation glucose should primarily be taken up in skeletal muscle and not into adipose tissue to be stored as triglycerides.

In conclusion, CART is expressed in both human and rat adipocytes and has both insulin-like and insulin-antagonistic effects on adipocytes. The importance of the modulatory effects of CART on adipocyte signaling and the role of CART actions in obesity remains to be elucidated.

5. Acknowledgements

6. References

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Figure legends

Figure 1. CART mRNA and peptide is expressed in rat and human subcutaneous and visceral adipose tissue.

RT-PCR was used to detect CART mRNA expression in adipose tissue. All the PCR products had the appropriate size on GelRed stained 1% agarose gel. CART: cocaine- and amphetamine-regulated transcript, NTC: non-template control, INS-1: INS-1 (832/13) cells, sc: subcutaneous adipose tissue, vis: visceral adipose tissue, sc-ln: subcutaneous adipose tissue from lean patient, sc-ob: subcutaneous adipose tissue from obese patient, om-ob(m): omental adipose tissue from obese male patient, om-ob(f): omental adipose tissue from obese female patient.

Immunofluorescence for CART (green) in human subcutaneous (**A, C**) and rat visceral (**B, D**) white adipose tissue. DAPI (blue) used in C and D to visualize nuclei. **E**: human subcutaneous white adipose tissue with abundant immunofluorescence for CART. **F**: Negative control staining of the same area as in **E** in a consecutive section using antibodies preabsorbed with CART 55-102. Scale bars: in A (for A-B) = 100µm; in F (for C-F) = 50µm.

Figure 2. CART potentiates isoprenaline-induced lipolysis and isoprenaline-induced activation of HSL.
A. Lipolysis was measured in primary rat adipocytes after stimulation for 30 min as indicated (Iso 30 nM: n=10, p=1.32E-08, Iso+CART 1 nM: n=5, p=0.049, 10 nM: n=5, p=0.052, 100 nM: n=10, p=0.015). **B.** Western blot analysis was performed with an antibody specific to phospho-HSL (Ser 563) on lysates originating from primary rat adipocytes after stimulation for 10 min as indicated (CART 100 nM, Iso 10-30 nM). Immunoblots from 8 separate experiments were quantified (CART: p=0.035, Iso 30 nM: p=0.029 and Iso+CART 100 nM: p=0.003) and representative blots are shown. CART: cocaine- and amphetamine-regulated transcript, Ctrl: control, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, HSL: hormone sensitive lipase, Iso: isoprenaline

Figure 3. CART potentiates insulin-induced inhibition of lipolysis and activates PDE3B.
A. Lipolysis was measured in primary rat adipocytes after stimulation for 30 min as indicated (Iso 30 nM: p=0.002, Iso+Ins 1 nM: p=0.038 and Iso+Ins+CART 100 nM: p=0.043, n=5). **B.**

PDE-activity was measured in the membrane containing pellet after centrifugation of homogenates originating from primary rat adipocytes, stimulated for 10 min as indicated (CART 100 nM: p=0.033 and Ins 1 nM: p=0.05, n=5). CART: cocaine- and amphetamine-regulated transcript, Ctrl: control, Ins: insulin, Iso: isoprenaline, PDE: phosphodiesterase

Figure 4. CART inhibits insulin-induced glucose uptake and PKB activation as well as insulin-induced lipogenesis.

A. Glucose uptake was measured after stimulating primary rat adipocytes for 30 min as indicated (Ins 1 nM: p=0.021 and CART 100 nM: p=0.026, n=5). **B.** Western blot analysis was performed with an antibody specific to phospho-PKB (Ser 473) on lysates originating from primary rat adipocytes after stimulation for 10 min as indicated (CART 100 nM, Ins 0.3-1 nM). Immunoblots from 6 separate experiments were quantified (Ins: p=0.02 and Ins+CART: p=0.005) and representative blots are shown. **C.** Lipogenesis was measured after stimulating primary rat adipocytes for 30 min as indicated (Ins 1 nM: p=0.0013, CART 100 nM: p=0.028, n=4) CART: cocaine- and amphetamine-regulated transcript, Ctrl: control, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, Ins: insulin, PKB: protein kinase B