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COCAINE- AND AMPHETAMINE-RELATED TRANSCRIPT (CART) DIRECTLY STIMULATES UCP-1 EXPRESSION AND ENHANCES INSULIN-INDUCED GLUCOSE UPTAKE

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ABSTRACT: *The hypothalamic cocaine- and amphetamine-regulated transcript (CART) is an anorexigenic neuropetide that inhibits food intake and increases thermogenesis in vivo via activation of the sympathetic nervous system. Apart from the central nervous system, CART expression can be found in peripheral tissues implicated in the regulation of food intake such as the gastrointestinal tract and the pancreas. Furthermore, CART circulates in the blood. Here, we report direct effects of CART on adipocyte functions. CART directly induced UCP-1 protein expression in brown adipocytes. This effect was time-dependent with a maximum 100% increase after 8 h ($p<0.01$). Furthermore, CART acutely enhanced insulin-induced glucose uptake by 25% ($p<0.01$). Accordingly, on a molecular level, acute CART stimulation increased the phosphorylation of a major proximal insulin signaling element such as protein kinase B by 80% ($p<0.05$). In contrast to these acute effects, CART did not affect adipocyte differentiation. Taken together, our results demonstrate a direct differentiation-independent effect of CART on adipocyte signaling and metabolism. They suggest a novel complementary role for this weight-regulatory neuropeptide in the peripheral control of energy homeostasis.*

KEY WORDS: Adipocyte, CART, Insulin sensitivity, Thermogenesis

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INTRODUCTION

The cocaine- and amphetamine-regulated transcript (CART) has been reported to play a critical role in the regulation of food intake and body weight. In humans (del Giudice *et al.*, 2001) and rodents (Asnicar *et al.*, 2001), mutations in or disruptions of the CART gene have been implicated in increased body weight, food intake, and fat mass. CART is one of the most abundant hypothalamic transcripts (Gautvik *et al.*, 1996). It inhibits food intake (Kristensen *et al.*, 1998; Thim *et al.*, 1998). However, mice expressing CART as a transgene display hyperphagia and become obese on a high-fat diet (Asnicar *et al.*, 2001). Within the hypothalamus, CART is colocalized with other anorexigenic and orexigenic peptides like proopiomelanocortin (POMC), the precursor of alpha MSH (Elias *et al.*, 1998), neuropeptide Y (NPY), and melanin-concentrating hormone (MCH) (Broberger *et al.*, 1999). A strong regulator of CART is leptin. Ob/ob mice show low CART mRNA levels, which increase after leptin administration (Kristensen *et al.*, 1998). CART also appears to increase energy expenditure (Hunter *et al.*, 2004). Thus, injection of CART into the paraventricular nucleus increases thermogenesis in brown adipocytes by stimulating UCP-1 gene expression (Wang *et al.*, 2000). CART is also expressed in peripheral organs including the gut, the adrenal gland, and the pancreas (Kuhar and Dall Vechia, 1999; Kuhar *et al.*, 2002; Ekblad *et al.*, 2003; Wierup *et al.*, 2004) and circulates in the blood (Jensen *et al.*, 1999). However, little information exists about peripheral actions of CART. Here, we report direct effects of CART on adipocyte signaling and metabolism. Thus, CART directly stimulates expression of the thermogenic uncoupling protein-1 (UCP-1) and enhances insulin signaling and action.

MATERIALS AND METHODS

Materials

Rat CART 55-102 was purchased from Bachem Distribution Services GmbH (Weil am Rhein, Germany). Phospho-specific antibodies directed against PKB (Ser473) were obtained from Cell signalling Technology, Inc. (Beverly, MA, USA). Antibodies directed against uncoupling protein-1 were from Chemicon International (Temecula, CA, USA). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co. (St Louis, Mo, USA).

Cell culture, SDS-PAGE and Western blotting

SV 40T-immortalized brown adipocytes were cultured

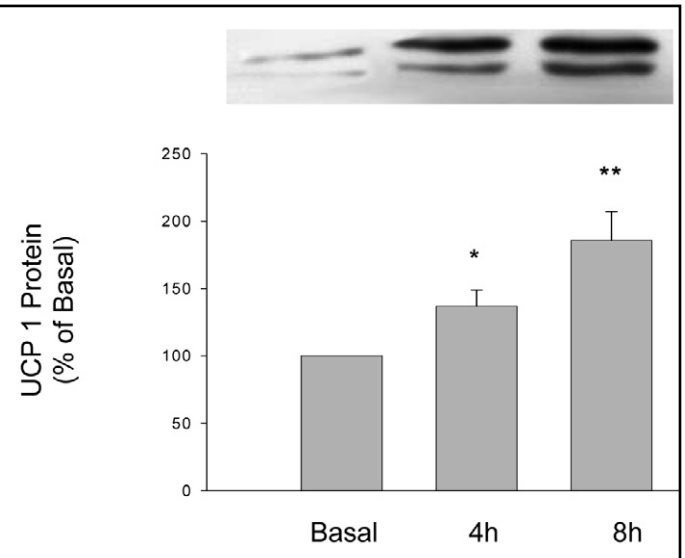

Figure 1: CART induces UCP-1 protein expression. Fully differentiated adipocytes were treated for 4 h or 8 h with 1 μM CART. UCP-1 protein expression was measured by immunoblotting using UCP-1 specific antibodies. A bar graph analysis of 5 independent experiments is shown.* denotes $p < 0.05$ and **$p < 0.01$ comparing CART-treated with unstimulated controls.

and differentiated as previously described (Klein *et al.*, 1999; Klein *et al.*, 2002). After reaching confluence, cell differentiation was induced by adding 250 μM indomethacin, 500 μM isobutylmethylxanthine and 2 μg/ml dexamethasone for 24 h to the differentiation medium. Fully differentiated adipocytes were starved for 24 h in serum-free medium before carrying out the experiments. After treatment with CART as indicated, cells were lysed, and protein levels were normalized prior to SDS-PAGE and immunoblotting. Bands were visualized using enhanced chemiluminescence (Roche Molecular Biochemicals, Mannheim, Germany).

Glucose uptake

Analysis of intracellular glucose uptake was performed in triplicate measurements in 12-well plates essentially as described previously (Klein *et al.*, 1999). In brief, fully differentiated cells were treated with CART, insulin or with both for the indicated periods of time. 50 μl of 2-deoxy-[^{3}H]glucose (0.5 μCi/ml final) were added for 4 additional minutes. The cells were washed in ice-cold phosphate-buffered saline and lysed with 0.1% SDS. The incorporated radioactivity was determined by liquid scintillation counting.

Oil Red O Staining

Cells were washed with PBS and fixed with 10% formalin for 30 minutes. After removing the formalin, 10 ml of the Oil Red O staining solution, previously filtered (Schleicher & Schuell GmbH; Dassel, Germany), were added for 1 h. To remove excess stain and any precipitate that may have formed, the cells were rinsed several times with distilled water.

Statistical analysis

Unpaired student's t-test was performed for determining statistical significance of differences using "sigma plot"-software (SPSS Science; Chicago IL, USA). P values <0.05 are considered significant, those <0.01 highly significant.

RESULTS

CART induces UCP-1 expression.

Thermogenesis is mediated by UCP-1 expression in brown adipocytes. Direct exposure of fully differentiated adipocytes to 1 μM CART stimulated UCP-1 protein expression. 8 h of CART treatment induced a 1.9 fold increase in UCP-1 protein expression as compared to non-treated cells ($p < 0.01$) (Fig. 1). This effect was time-dependent and already detectable after 4 h ($p < 0.05$) (Fig. 1). Furthermore, it did not appear to be associated with a general effect on protein synthesis since protein amounts of signaling molecules such as p70S6 were not affected by CART stimulation (data not shown). In accordance with the increase in UCP-1 protein expression, mRNA levels were upregulated by 25% after 8 h of CART stimulation (data not shown).

CART enhances insulin-induced glucose uptake.

Thermogenesis is linked to glucose uptake. Glucose uptake represents an important end point of the insulin signaling cascade. Insulin treatment (100 nM) induced a 5-fold increase of glucose uptake as compared to basal levels (Fig. 2). CART treatment alone (1 μM) did not have any effect on basal, non-insulin-stimulated glucose uptake (Fig. 2). However, when cells were pretreated with CART for 4 to 8 h prior to stimulation with insulin, glucose uptake was enhanced by 25% as compared to insulin stimulation alone ($p < 0.05$) (Fig. 2). This effect was time-dependent with a maximum after 4 h CART pretreatment ($p < 0.01$) (Fig. 2).

CART stimulates PKB phosphorylation.

PKB is an important proximal insulin signaling element and a main mediator of metabolic insulin effects. CART treatment (1 μM) of differentiated cells for 5 and 10 min increased PKB phosphorylation by approximately 30% and 80% ($p < 0.05$) (Fig. 3). However, CART did not enhance insulin-stimulated PKB phosphorylation (data not shown).

CART does not affect adipocyte differentiation.

Adipocyte responses such as UCP-1 expression and glucose uptake are differentiation-dependent. To disentangle potential differentiation-dependent effects from direct acute and subchronic effects of CART, we tested

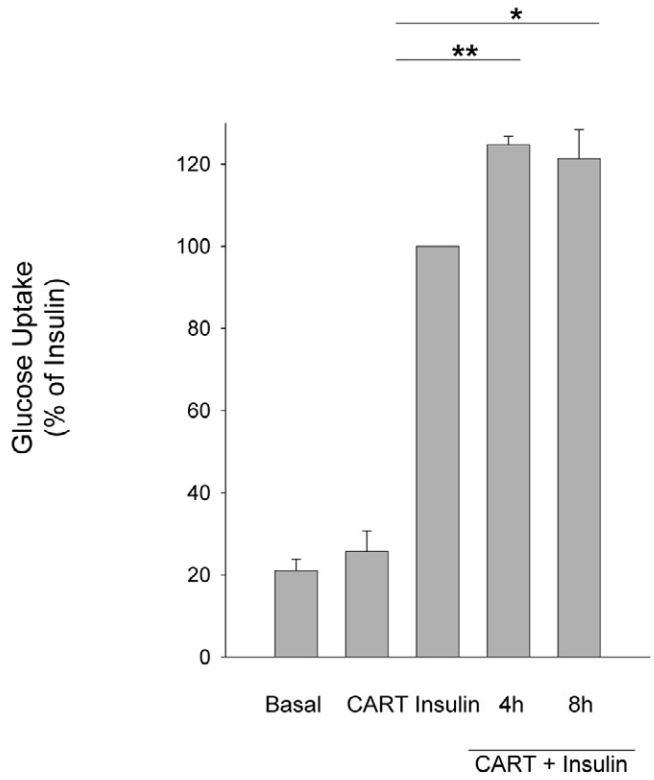

Figure 2: CART enhances insulin-induced glucose uptake. Cells were starved in serum-free medium 24 h before pretreatment with 1 µM CART for 4 h or 8 h. Intracellular glucose uptake was assessed after a 30 min period either without (Basal and for CART alone) or with insulin as indicated. Uptake of 3H-2-DOG, added 4 min before lysing the cells, was measured in a beta scintillation counter. Bars represent the mean of 7 independent experiments using triplicate measurements. *$p < 0.05$ and **$p < 0.01$ comparing CART-pretreated cells with insulin stimulation alone.

the influence of chronic CART exposure on adipocyte differentiation. Chronic treatment with 1 µM CART from the end of the induction period for the next 6 days did not affect adipocyte differentiation as assessed by the fat-specific oil red O staining (Fig. 4) and microscopically (data not shown).

DISCUSSION

CART is known as a neuropeptide with an important role in appetite control and maintenance of energy homeostasis. Recently, a significant body of literature has been accumulating on its central nervous system regulation and actions (Hunter *et al.*, 2004). CART is closely regulated by leptin, appears to predominantly act as an anorexigenic agent, and also stimulates energy expenditure, most likely via activation of the sympathetic nervous system. Although this peptide is expressed in peripheral tissue (Kuhar and Dall Vechia, 1999; Kuhar *et al.*, 2002; Ekblad *et al.*, 2003; Wierup *et al.*, 2004) and circulates in the blood (Jensen *et al.*, 1999; Vrang *et al.*, 1999; Vicentic *et al.*, 2004; Vicentic *et al.*, 2005), little is known about its peripheral actions. Our study demonstrates direct effects of this neuropeptide on adipose tissue functions. The stimulatory effects of CART on UCP-1 expression are consistent with its central nervous system actions favoring a negative energy balance. In fact, UCP-1 expression has been shown to be increased after injection of CART into the arcuate (Kong *et al.*, 2003) and paraventricular nuclei (Wang *et al.*, 2000) of mice. Our findings suggest a complementary direct mode of action in brown adipose tissue. However, it has to be kept in mind that the concentrations used in this *in-vitro* study are supraphysiologic, most likely with respect to both situations conceivable *in vivo*, endocrine and paracrine modes of neuropeptide action.

Brown adipose tissue (BAT) plays an important role in maintaining energy homeostasis in small animals (Cannon and Nedergaard, 2004). Apart from a potential effect of BAT-mediated thermogenesis on the total energy balance in humans, which remains a matter of debate (Klaus, 2004), a recent study also suggests a role for this tissue in regulating insulin sensitivity in humans (Yang *et al.*, 2003). We find a direct enhancing effect of CART on insulin-induced glucose uptake in adipocytes. In parallel, important insulin signaling intermediates are stimulated acutely by direct CART treatment. Interestingly, the kinetics of this functional response are different from the effect on thermogenesis, with an enhancement of glucose uptake preceding the maximum effect on UCP-1 expression. From a biological point of view, this may be in line with the notion that an increase in insulin sensitivity of brown adipocytes secures the energy influx required to fulfill their thermogenic function.

Finally, our findings argue for a differentiation-independent effect of CART on metabolic functions of fully differentiated cells, since chronic CART treatment does not affect the cellular phenotype and lipid accumulation.

Taken together, our findings provide molecular and functional evidence that direct peripheral actions of the anorexigenic neuropeptide CART influence expression of the thermogenic UCP-1 and insulin sensitivity. These effects may contribute to this peptide's biological role in the maintenance of energy homeostasis.

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REFERENCES

Asnicar, M.A., Smith, D.P., Yang, D.D., Heiman, M.L., Fox, N., Chen, Y.F., Hsiung, H.M. and Koster, A. (2001) Absence of cocaine- and amphetamine-regulated transcript results in obesity in mice fed a high caloric diet. *Endocrinology* **142**, 4394-4400.

Broberger, C., Holmberg, K., Kuhar, M.J. and Hokfelt, T. (1999) Cocaine- and amphetamine-regulated transcript in the rat vagus nerve: A putative mediator of cholecystokinin-induced satiety. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 13506-13511.

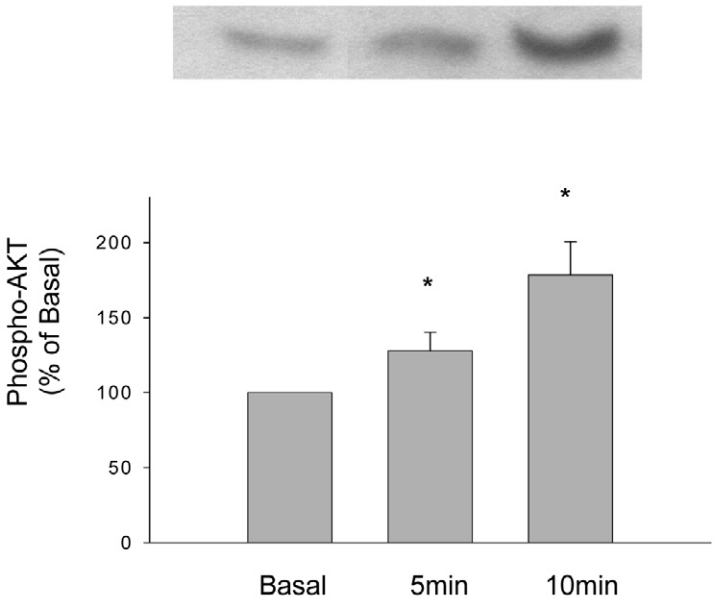

Figure 3: CART stimulates PKB phosphorylation.
Following starvation for 24 h in serum-free medium, cells were incubated for the indicated times with 1 μM CART. Protein lysates were prepared, and immunoblotting was performed. Phosphorylation of PKB was analyzed by direct immunoblotting using a phosphospecific antibody. A representative immunoblot and bar graph analysis including the S.E.M of four independent experiments are shown. *$p < 0.05$ comparing non-stimulated to CART-treated cells.

Cannon, B. and Nedergaard, J. (2004) Brown adipose tissue: function and physiological significance. *Physiological Reviews* **84**, 277-359.

del Giudice, E.M., Santoro, N., Cirillo, G., D'Urso, L., Di Toro, R. and Perrone, L. (2001) Mutational screening of the CART gene in obese children: identifying a mutation (Leu34Phe) associated with reduced resting energy expenditure and cosegregating with obesity phenotype in a large family. *Diabetes* **50**, 2157-2160.

Ekblad, E., Kuhar, M., Wierup, N. and Sundler, F. (2003) Cocaine- and amphetamine-regulated transcript: distribution and function in rat gastrointestinal tract. *Neurogastroenterology and Motility* **15**, 545-557.

Elias, C.F., Saper, C.B., Maratos-Flier, E., Tritos, N.A., Lee, C., Kelly, J., Tatro, J.B., Hoffman, G.E., Ollmann, M.M., Barsh, G.S., Sakurai, T., Yanagisawa, M. and Elmquist, J.K. (1998) Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *The Journal of Comparative Neurology* **402**, 442-459.

Gautvik, K.M., de Lecea, L., Gautvik, V.T., Danielson, P.E., Tranque, P., Dopazo, A., Bloom, F.E. and Sutcliffe, J.G. (1996) Overview of the most prevalent hypothalamus-specific mRNAs, as identified by directional tag PCR subtraction. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 8733-8738.

Hunter, R.G., Philpot, K., Vicentic, A., Dominguez, G., Hubert, G.W. and Kuhar, M.J. (2004) CART in feeding and obesity. *Trends in Endocrinology and Metabolism* **15**, 454-459.

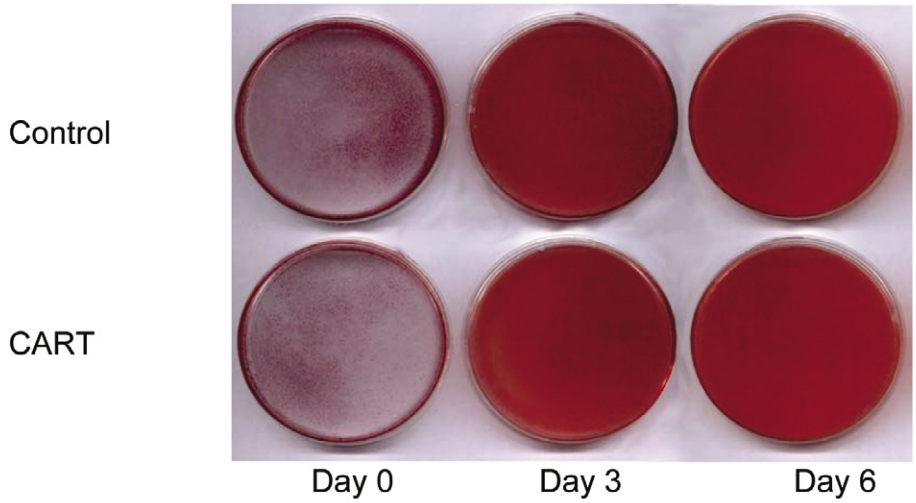

Figure 4: CART does not affect adipocyte differentiation.
Cells were exposed to 1 μM CART from the end of the induction period. Medium was changed every 24 h. At the days indicated, cells were stained with the fat-specific Oil red O solution.

Jensen, P.B., Kristensen, P., Clausen, J.T., Judge, M.E., Hastrup, S., Thim, L., Wulff, B.S., Foged, C., Jensen, J., Holst, J.J. and Madsen, O.D. (1999) The hypothalamic satiety peptide CART is expressed in anorectic and non-anorectic pancreatic islet tumors and in the normal islet of Langerhans. *FEBS letters* **447**, 139-143.

Klaus, S. (2004) Adipose tissue as a regulator of energy balance. *Current Drug Targets* **5**, 241-250.

Klein, J., Fasshauer, M., Ito, M., Lowell, B.B., Benito, M. and Kahn, C.R. (1999) beta(3)-adrenergic stimulation differentially inhibits insulin signaling and decreases insulin-induced glucose uptake in brown adipocytes. *The Journal of Biological Chemistry* **274**, 34795-34802.

Klein, J., Fasshauer, M., Klein, H.H., Benito, M. and Kahn, C.R. (2002) Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action. *Bioessays* **24**, 382-388.

Kong, W.M., Stanley, S., Gardiner, J., Abbott, C., Murphy, K., Seth, A., Connoley, I., Ghatei, M., Stephens, D. and Bloom, S. (2003) A role for arcuate cocaine and amphetamine-regulated transcript in hyperphagia, thermogenesis, and cold adaptation. *The FASEB Journal* **17**, 1688-1690.

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

Kuhar, M.J. and Dall Vechia, S.E. (1999) CART peptides: novel addiction- and feeding-related neuropeptides. *Trends in Neurosciences* **22**, 316-320.

Kuhar, M.J., Adams, S., Dominguez, G., Jaworski, J. and Balkan, B. (2002) CART peptides. *Neuropeptides* **36**, 1-8.

Thim, L., Kristensen, P., Larsen, P.J. and Wulff, B.S. (1998) CART, a new anorectic peptide. *The International Journal of Biochemistry & Cell Biology* **30**, 1281-1284.

Vicentic, A., Dominguez, G., Hunter, R.G., Philpot, K., Wilson, M. and Kuhar, M.J. (2004) Cocaine- and amphetamine-regulated transcript peptide levels in blood exhibit a diurnal rhythm: regulation by glucocorticoids. *Endocrinology* **145**, 4119-4124.

Vicentic, A., Hunter, R.G. and Kuhar, M.J. (2005) Effect of corticosterone on CART peptide levels in rat blood. *Peptides* **26**, 531-533.

Vrang, N., Tang-Christensen, M., Larsen, P.J. and Kristensen, P. (1999) Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour. *Brain Research* **818**, 499-509.

Wang, C., Billington, C.J., Levine, A.S. and Kotz, C.M. (2000) Effect of CART in the hypothalamic paraventricular nucleus on feeding and uncoupling protein gene expression. *Neuroreport* **11**, 3251-3255.

Wierup, N., Kuhar, M., Nilsson, B.O., Mulder, H., Ekblad, E. and Sundler, F. (2004) Cocaine- and amphetamine-regulated transcript (CART) is expressed in several islet cell types during rat development. *The Journal of Histochemistry and Cytochemistry* **52**, 169-177.

Yang, X., Enerback, S. and Smith, U. (2003) Reduced expression of FOXC2 and brown adipogenic genes in human subjects with insulin resistance. *Obesity Research* **11**, 1182-1191.