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Expression analysis of a cholecystokinin system in human and rat white adipose tissue

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ABSTRACT

Aim: Cholecystokinin (CCK) participates in the storage of dietary triglycerides in white adipose tissue (WAT). Our goal was to characterize, both in subcutaneous (Sc-WAT) and visceral WAT (Vis-WAT), the functional expression of the two known CCK receptors, CCK-1 (CCK-1R) and CCK-2 (CCK-2R), as well as of CCK.

Main methods: Gene and protein expression was assessed in different cell types of rat and human WAT by means of RT-PCR and western-blot, respectively. The functionality of CCK-Rs was tested by quantifying protein kinase B (Akt) phosphorylation after treatment of pre-adipocytes with the bioactive fragment of CCK, CCK-8. The CCK receptor subtype involved in Akt phosphorylation was investigated by using selective CCK-1R (SR-27,897) and CCK-2R antagonists (L-365,260).

Key findings: In rats, CCK-1R (*Cckar*) and CCK-2R (*Cckbr*) gene expression was detected in the two types of WAT analyzed as well as in isolated adipocytes, mesenchymal stem cells and pre-adipocytes. CCK-1R and CCK-2R proteins were identified in adipocytes and, to a minor extent, in pre-adipocytes. In addition, CCK-2R were detected in subcutaneous mesenchymal stem cells. Gene expression of the CCK precursor preproCCK as well as CCK immunoreactivity were also found in Sc-WAT and Vis-WAT. In human WAT, *CCK* gene expression as well as CCK-2Rs and CCK were also identified. CCK-8 evoked Akt phosphorylation in rat pre-adipocytes, and this effect was antagonized by SR-27,897 and L-365,260.

Significance: Our data show that both human and rat WAT express a complete CCK system, and suggest that CCK may have an autocrine/paracrine role in regulating adipose tissue biology.

1. Introduction

Cholecystokinin (CCK) is a pleiotropic gut hormone. In addition to its well-defined action in stimulating the release of pancreatic lipase, that allows intestinal digestion of fat and further triglyceride (TG) absorption, CCK inhibits gastric emptying and promotes short-term satiety by acting on CCK-1 receptors (CCK-1R) located in abdominal vagal afferences as well as in brainstem areas [1]. In addition, recent research has suggested that CCK is a link between the intestine and white adipose tissue (WAT), as fatty acid (FA) uptake by adipose tissue appears to be impaired in *Cck* knockout mice [2]. In this regard, our group has demonstrated that CCK promotes the incorporation of circulating TGs to WAT by activating lipoprotein lipase (LPL). The mechanism that accounts for this CCK effect concerns the expression of angiopoietin-like protein 4 (ANGPTL-4) [3], a molecular chaperone that binds and inactivates LPL [4]. These findings suggests that CCK and CCK-Rs might be relevant in regulating the expandability of WAT and, therefore, in modulating energy balance.

The adipose tissue is a tissue endowed with endocrine functions that produces the so-called adipokines, a heterogeneous family of peptide/protein hormones endowed with multiple physiological functions related with energy balance regulation, such as food intake, adipogenesis, catabolic activity in oxidative tissues, and insulin responsiveness [5]. Adipokines behave as endocrine mediators whose physiological plasma concentration mostly depend on adiposity levels, but are also sensitive to physiopathological conditions such as fasting, obesity, and diabetes

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[6]. The widespread expression of adipokine receptors covers the brain and peripheral tissues, including WAT; for instance, leptin [7], adiponectin [8], as well as apelin receptors [9] appear to be functionally expressed in WAT. Thus, adipokines, together with their corresponding receptors, conform local systems that account for both the autocrine and paracrine control of WAT.

On this basis we have hypothesized that a CCK system may be integral to regulatory circuits in WAT. The current study aimed at characterizing i) the expression pattern of CCK and CCK-Rs in both rat and human WAT, and i) the ability of CCK-Rs to phosphorylate/activate Akt, a protein kinase integral to different signal transduction pathways pivotal to maintain adipocyte homeostasis.

2. Material and methods

2.1. Chemicals

The CCK-1R antagonist, SR-27,897 (1-[2r-(2-chlorophenyl)thiazol-2-yl-aminocarbo nyl]-indolylacetic acid), was kindly provided by Sanofi-Synthelabo, France [10]. The CCK-2R antagonist, L-365,260 [(3R-(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzo diazepin-3-yl)-N′-(3-methylphenyl)urea], was a gift of Merck Sharp and Dohme Research Laboratories, USA [11]. Other chemicals were from Sigma (St. Louis, USA).

2.2. Animals

Experiments were conducted in 3-month old male Wistar rats, maintained under controlled light (12 h light cycles from 08:00 to 20:00) and temperature (22–24 °C) conditions in the animal facility of the Universidad San Pablo-CEU. The rats were fed a normal chow (3.1 kcal/g; A.04, Panlab, Barcelona, Spain) and had free access to tap water. Animals were decapitated under deep isoflurane anaesthesia, and subcutaneous (Sc-WAT) and perirenal (Vis-WAT) adipose tissues were rapidly removed. The studies presented here conform to the Guide for the Care and Use of Laboratory Animals (European Communities Council Directive 86/609/EEC) and were approved by the Ethics Committee of the Universidad San Pablo-CEU.

2.3. Human adipose tissue sampling

Biopsy samples of omental and subcutaneous adipose tissue were obtained during bariatric surgery of 3 subjects. All participants provided written informed consent and study protocol and procedures were approved by the Ethical Committee of Hospital Clínico San Carlos (Madrid, Spain), according to the ethical standards of the Declaration of Helsinki.

2.4. Isolation of rat adipocytes and adipose stromal vascular cells, and differentiation of pre-adipocytes

The procedure was carried out as previously described [3,12]. Briefly, Vis-WAT and Sc-WAT were chopped and then incubated for 1 h in α-MEM containing 13.6 U/ml type NB4 collagenase (Serva, Germany) and 20 μg/ml type I DNAse (Roche, Spain). After enzymatic digestion, samples were centrifuged at 300*g* (10 min, 25 °C) and supernatants containing adipocytes preserved for further analysis. The pellets containing the stromal vascular fraction (SVF) were re-suspended in 1 ml α-MEM, then filtered through a 37 μm mesh and centrifuged at 300*g* (10 min, 25 °C). Pellets were treated with erythrocyte lysis buffer (155 mM NH_4Cl, 5.7 mM K_2HPO_4, 0.1 mM EDTA, pH = 7.3), then re-centrifuged under identical conditions, re-suspended in α-MEM supplemented with 10% newborn calf serum (NCS, Sigma, Spain), 10 U/ml penicillin-streptomycin (LifeTechnologies, Spain) and 0.25 μg/ml amphotericin B (Lifetechnologies, Spain), plated in 12-well plates (40.000 cells/cm^2) and cultured during 5–7 days. Differentiation of adipose-derived mesenchymal stem cells (ASC) into preadipocytes was induced in α-MEM containing 2% NCS, 66 nM insulin, 1 nM tri-iodothyronine, 1 mM dexamethasone, 0.3 mM rosiglitazone and 10 mg/ml apotransferrin (7–9 days).

2.5. Cholecystokinin receptor gene silencing

Differentiated rat pre-adipocytes were transfected with siRNAs for *Cckar* and *Cckbr* (s234619 and s218025, respectively; Life Technologies, Spain), as previously described [3]. Briefly, for each well, siRNAs (40 nmol/l) were diluted in 50 μl Opti-MEM (Life Technologies), incubated 15 min (25 °C) and then added to a solution containing 3 μl lipofectamine RNAiMAX (Life Technologies) diluted in 50 μl Opti-MEM. After 15 additional min (25 °C), 100 μl of the mixture were added drop-wise to wells containing 400 μl Opti-MEM. The medium was replaced by differentiation media after overnight incubation. Two days after transfection, the medium was changed to α-MEM containing 0.1% BSA, then CCK-8 (10^{-6} mol/l) was added and incubated for 2 h (37 °C, 5% CO_2). Media was removed and mRNA was extracted as detailed below. Positive controls for transfection were performed by using Block-iT Alexa Fluor Red (Life Technologies). A Silencer Select Negative Control (4,390,843, Thermo Fisher Scientific, Spain) was used.

2.6. Western-blot

Brain, WAT samples and cells were homogenized in ice-cold buffer containing 0.42 M NaCl, 20 mM HEPES (pH 7.9), 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 20 mM sodium fluoride, 1 mM trisodium orthovanadate, and 2 mM phenylmethylsulfonyl fluoride. The homogenates were frozen at −80 °C, thawed at 37 °C (×3) and then centrifuged for 10 min at 4 °C. Equal amounts of protein (50 μg) were loaded in Laemmli buffer (50 mM Tris pH = 6.8, 10% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 2 mg/ml blue bromophenol), and size separated by SDS-PAGE. After protein transfer to PVDF membranes (GE Healthcare, Spain) and blocking with 5% nonfat dried milk/Tween-PBS (1 h), primary antibodies against human/rat CCK-1Rs and CCK-2Rs (sc-514,303 and sc-166,690, respectively; mouse monoclonal antibodies, Santa Cruz Biotechnology Inc. CA), human/rat anti-CCK (ab83189; rabbit polyclonal antibody directed against the 66–115 amino acid sequence of human CCK; Abcam, UK), phosphoAkt-Ser^{473} (9271, rabbit polyclonal antibody; Cell Signaling Technology Inc., Beverly, MA), and Akt (9272; rabbit polyclonal antibody; Cell Signaling Technology Inc., Beverly, MA), were applied at the convenient dilution (1:250 for CCK-Rs and 1:1000 for phosphoAkt-Ser^{473} and Akt antibodies) overnight at 4 °C. After washing, appropriate secondary antibodies (anti-mouse IgG-peroxidase or anti-rabbit IgG-peroxidase conjugated) were added for 1 h at a dilution of 1/5000. Blots were incubated in chemiluminescence reagents (ECL Prime, UK) and bands were detected by using the ChemiDoc XRS+ Imaging System (BioRad, Spain). CCK-1R and CCK-2R values were normalized to β-actin (β-actin antiserum, Affinity Bioreagents, Golden, CO). pAkt values were normalized with Akt. Brain tissue and pre-adipocytes in which *Cckar*/*Cckbr* gene expression was silenced were used as positive and negative controls, respectively.

2.7. Quantitative real-time PCR

Total RNA was extracted from brain, duodenum, liver and cells by using the Tri-Reagent protocol (LifeTechnologies, Spain). cDNA was then synthesized from 1 μg total mRNA by using a high-capacity cDNA RT kit (Applied Biosystems, USA). Quantitative RT-PCR was performed by using designed primer pairs (Integrated DNA Technologies, USA; Table 1). SsoAdvanced Universal SYBR Green Supermix (Biorad, Spain) was used for amplification according to the manufacturer's protocols in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems,