Molecular and Cellular Endocrinology 431 (2016) 101–108

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

An analogue of atrial natriuretic peptide (C-ANP$_{4-23}$) modulates glucose metabolism in human differentiated adipocytes

Francisco Javier Ruiz-Ojeda [a, b, c], Concepción María Aguilera [a, b, c, d], Azahara Iris Rupérez [a, b], Ángel Gil [a, b, c, d], Carolina Gomez-Llorente [a, b, c, d, *]

[a] Department of Biochemistry and Molecular Biology II, School of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain
[b] Institute of Nutrition and Food Technology "José Mataix", Center of Biomedical Research, University of Granada, Avda. del Conocimiento s/n, 18016 Armilla, Granada, Spain
[c] Instituto de Investigación Biosanitaria ibs, Granada, Spain
[d] CIBER Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Madrid, Spain

ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form
27 April 2016
Accepted 10 May 2016
Available online 13 May 2016

Keywords:
Obesity
Adipocytes
Natriuretic peptide
Natriuretic peptide receptor 3

ABSTRACT

The present study was undertaken to investigate the effects of C-atrial natriuretic peptide (C-ANP$_{4-23}$) in human adipose-derived stem cells differentiated into adipocytes over 10 days (1 μM for 4 h). The intracellular cAMP, cGMP and protein kinase A levels were determined by ELISA and gene and protein expression were determined by qRT-PCR and Western blot, respectively, in the presence or absence of C-ANP$_{4-23}$. The levels of lipolysis and glucose uptake were also determined. C-ANP$_{4-23}$ treatment significantly increased the intracellular cAMP levels and the gene expression of *glucose transporter type 4* (*GLUT4*) and *protein kinase, AMP-activated, alpha 1 catalytic subunit (AMPK)*. Western blot showed a significant increase in GLUT4 and phosphor-AMPKα levels. Importantly, the adenylate cyclase inhibitor SQ22536 abolished these effects. Additionally, C-ANP$_{4-23}$ increased glucose uptake by 2-fold. Our results show that C-ANP$_{4-23}$ enhances glucose metabolism and might contribute to the development of new peptide-based therapies for metabolic diseases.

1. Introduction

Obesity and its comorbidities, specifically metabolic syndrome and type 2 diabetes, have reached epidemic proportions, especially in developed countries. Natriuretic peptides (NPs) have been revealed to be key mediators of metabolic processes and have been implicated in the development of diabetes (Gurden et al., 2014). NPs are a family of peptide hormones that are predominantly secreted from the heart and that exert a variety of physiological functions by interacting with NP receptors (NPRs) (Li et al., 2014, Schlueter et al., 2014). The NP family comprises three members, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). These peptides are secreted as pro-hormones and are then cleaved by proteases into their biologically active forms and corresponding inactive peptides at equimolar ratios. There are three NPRs: NPR1 and NPR2 are membrane guanylyl cyclase receptors that are primarily responsible for the metabolic activity of NPs, and NPR3, a non-guanylyl cyclase-coupled receptor (Levin, 1993), has been classically considered as a clearance receptor that is involved in the degradation of NPs (Schlueter et al., 2014). NPR3 can bind to all NPs; it displays the highest affinity for ANP and the lowest affinity for BNP (Potter et al., 2006).

NPRs are expressed in different human tissues, including adipose tissue, where NPs can stimulate lipolysis, modulate adipokine secretion, and promote white adipocyte browning (Gurden et al., 2014; Sarzani et al., 1996; Moro and Lafontan, 2013; Bordicchia et al., 2012). Additionally, the expression levels of *NPR3* in

Abbreviations: AMPK, AMP-activated kinase; BSA, bovine serum albumin; CASP1, caspase 1; C-ANP$_{4-23}$, C-atrial natriuretic peptide (4-23); cAMP, cyclic adenosine monophosphate; CNP-22, C-type natriuretic peptide-22; cGMP, cyclic guanosine monophosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; HPRT1, hypoxanthine-guanine phosphoribosyltransferase-1; IL1B, interleukin 1, beta; NPR3, natriuretic peptide receptor 3; PKA, protein kinase A.

* Corresponding author. Department of Biochemistry and Molecular Biology II, Institute of Nutrition and Food Technology "José Mataix", Center of Biomedical Research, University of Granada, Avda. del Conocimiento s/n, 18016 Armilla, Granada, Spain.
E-mail address: gomezll@ugr.es (C. Gomez-Llorente).

http://dx.doi.org/10.1016/j.mce.2016.05.011
0303-7207/

adipose tissue have been observed to be considerably increased in obese adults (Gómez-Ambrosi et al., 2004; Baranova et al., 2005) and obese children (Aguilera et al., 2015). Moreover, insulin increases *NPR3* expression in subcutaneous adipose tissue in subjects with moderate obesity and normal glucose tolerance (Pivovarova et al., 2012). Several studies have revealed that the plasma levels of NPs are decreased in patients with obesity or type 2 diabetes (Moro and Lafontan, 2013, Wang et al., 2007) and this decrease may be primarily due to impaired NP release (Omar et al., 2009). Another possible explanation is that the up-regulation of *NPR3* increases NP clearance from the circulation (Dessì-Fulgheri et al., 1997) although the ablation of *NPR3* in mice did not affect the circulating levels of NPs (Pivovarova et al., 2012, Matsukawa et al., 1999). It has been reported that NPR3 is coupled to the adenylate cyclase/cAMP system through a 37-amino acid intracellular region of NPR3 that is expressed in various human tissues (Schenk et al., 1987, Sengenes et al., 2005). Studies using the synthetic ligand C-ANP$_{4–23}$, a ring-deleted analogue of ANP, that specifically interacts with NPR3, have shown decreased cAMP levels in the anterior pituitary, the aorta, the brain striatum, and the adrenal cortex, as well as in rat vascular smooth muscle cells and cultured adipocytes (Anand-Srivastava et al., 1990; Crilley and Garcia, 1997). However, incubation in C-ANP$_{4-23}$ increased the cAMP levels in human thyrocytes (Sellitti et al., 2001). In addition, in cultured adipocytes, molecules that increase the intracellular cAMP levels have been demonstrated to increase the activity of AMP-activated protein kinase (AMPK), a critical regulator of energy homeostasis and a therapeutic target for the treatment of type 2 diabetes (Yin et al., 2003, Hutchinson et al., 2005). In human adipocytes, the modulation of the cAMP levels by C-ANP$_{4-23}$ has not been reported. Consequently, the present study was undertaken to determine whether the C-ANP$_{4-23}$ treatment alters glucose metabolism via AMPK in human adipocytes and to explore whether this effect is mediated by the adenylate cyclase system.

2. Material and methods

2.1. Materials

Commercially available adipose derived-stem cells (ADSCs) were purchased directly from Lonza (Poietics™ Normal ADSCs, Lonza, *PT-5006,* Lot 0F4505, Switzerland). ADSCs are isolated from normal (non-diabetic) adult lipoaspirates collected during elective surgical liposuction procedures (from subcutaneous adipose tissue). ADSCs have been reported to differentiate down many different lineages including chondrogenic, osteogenic, adipogenic and neural. ADSCs have been cryopreserved at primary passage. Adipogenesis media were obtained from Lonza. Oil Red O was acquired from Sigma (234117, Sigma-Aldrich, St. Louis, MO, USA). C-ANP$_{4–23}$ (H3134), ANP (H2095) and CNP-22 (H1296) were purchased from Peninsula Laboratories (Bachem AG, Switzerland). An adenylate cyclase inhibitor (SQ22536, T2678) was acquired from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The rabbit anti-glucose transporter type 4 (GLUT4) antibody (H-61) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the rabbit anti-total AMPKα (D5A2) and rabbit anti-phospho-AMPKα (Thr172) antibodies were obtained from Cell Signalling Technologies (Beverly, MA, USA), and the mouse anti-α-tubulin antibody (T5158) and horseradish peroxidase-conjugated immunoglobulin were purchased from Sigma. Unless otherwise indicated, all other chemicals were purchased from Sigma.

2.2. Cell culture and incubation

ADSCs were cultured, expanded and differentiated into adipocytes according to the manufacturer's recommendations (Lonza). Briefly, ADSCs were grown and expanded in appropriate sterile plastic dishes in complete Advanced-DMEM (Gibco, Life Technologies, Spain) supplemented with 2 mM L-glutamine (25030, Gibco), 10% foetal bovine serum (FBS, PT-9000 H, Lonza), 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (10378-016, Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cell culture medium was replaced twice per week, and the cells were passaged a maximum of 6 times. To induce differentiation, the cells were seeded in 35-mm dishes at a density of 30,000 cells/cm^2 and cultured in preadipocyte growth medium (PGM) consisting of Preadipocyte Basal Medium-2 (PT-8002, Lonza) supplemented with 10% FBS, 2 mM L-Glutamine (PT-9001 H, Lonza) and 0.1 μg ml^{-1} Gentamicin Sulfate Amphotericin-B (PT-4504, Lonza). At 90% confluency, the growth medium was replaced with differentiation medium (PGM supplemented with dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), indomethacin and h-insulin, PT-9502, Lonza). ADSCs were differentiated for 10 days. Finally, the cells were washed twice with PBS, and the differentiation medium was replaced with PGM and IBMX (0.1 mM) overnight. All treatments were performed on differentiated adipocytes at day 10.

In order to test the appropriate concentration and incubation time of C-ANP$_{4-23}$, the cells were treated with different concentrations (50 nM, 1 μM and 5 μM) and incubation times (30 min, 4 h and 6 h). The selected experimental conditions, based on the intracellular cAMP levels results, were 1 μM of C-ANP$_{4-23}$ and 4 h of incubation (Supplementary Fig. S3). Subsequently, the cells were treated with 100 μM SQ22536 for 30 min, in the presence or absence C-ANP$_{4-23}$, as described below. The appropriate concentration and incubation time of SQ22536 were obtained from the available literature (Santangelo et al., 2011).

2.3. Intracellular cAMP and cGMP level determination

Human differentiated adipocytes were incubated in the presence or absence of C-ANP$_{4–23}$, ANP (1 μM, 4 h) or CNP-22 (1 μM, 4 h), and the intracellular cAMP and cGMP levels were determined using the commercial cAMP (ADI-900-163, Enzo Life Science, Switzerland) and cGMP (ADI-900-164, Enzo Life Science, Switzerland) ELISA kits. In additional experiments, the cells were incubated in SQ22536 prior to treatment with or without C-ANP$_{4–23}$, followed by determination of the intracellular cAMP levels using the aforementioned kit.

2.4. Protein kinase A (PKA) activity assay

Human differentiated adipocytes were incubated in the presence or absence of C-ANP$_{4–23}$, and Protein kinase A (PKA) activity was determined using the PKA kinase activity kit (ADI-EKS-390A, Enzo Life Science, Switzerland). Additionally, the cells were incubated in SQ22536 prior to treatment with or without C-ANP$_{4–23}$, followed by determination of PKA activity using the aforementioned kit.

2.5. Lipolysis assay

The total level of glycerol, the final product of lipolysis, was measured in cell supernatants using a colorimetric assay (Free Glycerol Reagent, F6428, Sigma-Aldrich, St. Louis, MO, USA). The cells were treated with 1 μM C-ANP$_{4–23}$ and with 1 μM ANP as a positive control or with CNP-22 (1 μM) during 4 h, and the cell supernatants were harvested. Then, the cell supernatants were incubated in the reagent at room temperature for 15 min in a 96-well plate, and the optical density was measured using a