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Original article

Obestatin changes proliferation, differentiation and apoptosis of porcine preadipocytes

L'obestatine influence la prolifération, la différentiation et l'apoptose des cellules préadipocytaires de porc

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Abstract

Obestatin, originally identified and purified from rat stomach extracts, was reported to bind to orphan G protein-coupled receptor, GPR39, and inhibit appetite and gastric motility. This study was conducted to investigate the effects of porcine obestatin on proliferation, differentiation and apoptosis of porcine preadipocytes isolated from subcutaneous fat of piglets. At indicated times of culture, morphology of preadipocytes and accumulated lipid droplets within the cells were identified by invert microscope. After treating with obestatin (0, 0.1, 1, 10 and 100 nM), cell proliferation was measured by MTT method and protein expression of CCAAT/enhancer binding protein-α (C/EBPα), peroxisome proliferator-activated receptor-γ (PPARγ), Caspase-7 and Caspase-9 was determined by Western Blot, mRNA expression of GPR39 and Caspase-3 was analyzed by RT-PCR, and the activity of Caspase-3 was measured by spectrophotometric method. The results showed that obestatin had no effect on GPR39 expression, while promotes the optical density (OD) value of cells, enhanced protein expression of PPARγ and C/EBPa, decreased mRNA expression and activity of Caspase-3, and inhibited protein expression of Caspase-7 and Caspase-9 in a dose-dependent manner. These results suggested that obestatin enhance proliferation and differentiation of preadipocytes promoting PPARγ and C/EBPa expression, and inhibiting preadipocyte apoptosis by decreasing expression of Caspase-3, Caspase-7 and Caspase-9.

1. Introduction

A number of clinical studies have demonstrated that adipose tissue is integrally involved in coordinating a variety of biological processes including energy metabolism, immune function, and neuroendocrine function [1]. The important endocrine function of adipose tissue is emphasized by the adverse metabolic consequences of both adipose tissue excess and deficiency [2]. Besides the biological repertoire necessary for storing and releasing energy, adipose tissue contains the metabolic machinery to permit communication with distant organs including the central nervous system (CNS). It is now clear that adipose tissue is a complex and highly active metabolic and endocrine organ [3,4]. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells [5]. The previous findings suggested that visceral fat accumulation played crucial roles in the development of cardiovascular disease as well as the development of obesity-related disorders such as hyperlipidemia, diabetes mellitus and the so-called metabolic syndrome [6]. Given these clinical findings, adipocytes functions have been intensively investigated in the past 10 years, and have been revealed to act as endocrine cells, which express and secrete several endocrine hormones such as leptin and adiponectin [7]. To present, most in vitro studies on adipocyte differentiation and metabolism have been performed using the murine preadipocyte cell line, 3T3-L1 and bovine intramuscular preadipocytes, which have enabled researchers to establish a great deal of knowledge on adipogenesis [8].

Obestatin, derived from *preproghrelin* gene and composed of 23 acid amino residues, is purified from stomach extracts and detected in peripheral plasma [9]. Obestatin was originally proposed to be the ligand for GPR39 [9], a receptor related to the ghrelin receptor subfamily, but this remains controversial. This novel peptide has shown to be multifunctional, which is involved in inhibiting thirst [10], regulating gastrointestinal functions, influence food intake and body weight gain [9,11], as well as improving sleeping [12] and memory [13]. Obestatin also plays

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an important role in the regulation of growth hormone release, appetite, and energy metabolism [14]. Recently, the findings have demonstrated that obestatin stimulate the proliferation of human retinal pigment epithelial and gastric cancer cells [15,16], prevents apoptosis in both rodent β-cells and human pancreatic islets by binding to specific obestatin receptors. In rat H9c2, cardiac cells or isolated ventricular myocytes subjected to I/R, 50 nmol/l obestatin-(1-23) reduced cardiomyocyte apoptosis and reduced Caspase-3 activation [17]. These suggest that obestatin may also play a role in cell survival [18]. However, obestatin and its effects on porcine preadipocytes are not fully elucidated. Therefore, to gain further insight into the influence of obestatin on porcine preadipocytes, in the present study, we have investigated the effects of obestatin on proliferation, differentiation and apoptosis of porcine preadipocytes. It may provide a base knowledge for our understanding of obestatin in regulating lipidosis and lipometabolism.

2. Materials and methods

2.1. Cell isolation and purification

Cell isolation and purification was done according to the methods described by Akanbi et al. [19]. Adipocytes were freshly isolated from samples of subcutaneous adipose tissue of Landrace piglets at 7 days old. Briefly, pieces of adipose tissue of piglets were incubated for 30 min at 37 °C with 0.1% (w/v) collagenase I containing 10 mM HEPES, 118 mM NaCl, 50 mM KCl, 1 mM $CaCl_2$, l5 mM D-glucose and 1.5% (w/v) BSA (Gibco, USA) in a sterile 50 mL plastic tube. Following digestion at 37 °C for 60–70 min with gentle shaking in a water bath, the suspension was filtered through sterile nylon mesh with 100 pores to remove undigested tissue and mature adipocytes and centrifugated at 800 × g for 5 min. After washing with DMEM, red cell lysate (154 mM NH_4Cl, 5.7 mM K_2HPO_4 and 0.1 mM EDTA) was added to remove red cells. The isolated and purified cells then were numbered and photographed for following analyses. All animals received human care in compliance with the guide for the care and use of experimental animals (Animal Care Committee, 2002).

2.2. Cell culture

Porcine preadipocytes were plated at a density of 1×10^5 cells/mL and grown in DMEM supplemented with 10% fetal bovine serum (FBS), and 100 U/mL penicillin and 100 mg/mL streptomysin at 37 °C under a humidified atmosphere containing 5% CO_2. Fresh medium without FBS was replenished every 24 h. In following studies, porcine preadipocytes were treated with 0, 0.1, 1, 10 and 100 nM porcine obestatin peptide (Phoenix, CA, USA) to study cell proliferation, differentiation and apoptosis.

2.3. Preadipocyte identification

Porcine preadipocytes in primary cultures were identified through visualizing the morphology and their functions conversing into adipocytes. At 24 h, cells were rinsed three times in 0.01 mmol/L PBS and then fixed in trypan blue for 10 min. Subsequently, the fixed cells were rapidly rinsed with PBS for 5 times and visualized. The cells at indicated times in culture were also visualized by inverted microscope (Olympus, Japan). The preadipocytes and their conversion into adipocytes were confirmed by the detection of lipid droplets under microscopy using oil red O staining which was given a detail presentation in section of oil red O staining.

2.4. MTT method

The endogenous effect of obestatin peptide on cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, the preadipocytes isolated and identified above were seeded in 96-well plates at a density of 1.0×10^5/mL in DMEM containing 10% FBS for 24 h, then the medium was changed and the cells were cultured in a serum-free medium. After incubation for 24 h, cells were incubated with different exogenous concentrations of the peptide (0, 0.1, 1, 10 and 100 nM) in fresh DMEM. At 24 h, 72 h and 120 h, cell proliferation was evaluated by adding 15 μl 5 mg/mL methyl thiazolyl tetrazolium (MTT; Sigma-Aldrich, USA) solution to each well of one cell culture plate, and further incubating for 4 h. Then the medium was removed, and 150 μl dimethyl sulphoxide was added to each well, and the plate was agitated for 10 min on a shaker to dissolve formazan. The 490 nm absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.5. Oil red O staining

In order to determine the extent of preadipocyte differentiation, Oil Red O staining were used in this study [20]. The preadipocytes were seeded in 96-well plates at a density of 1.0×10^4 cells per well in DMEM containing 10% FBS for 24 h, and 0, 0.1, 1, 10 and 100 nM obestatin was added to the serum-free medium once cells were confluent and had begun to differentiate. At indicated times, the medium was removed, and cells were washed three times with phosphate-buffered saline (PBS). After washing three times with PBS, cells were stained for at least 10 min with 1% filtered oil red O (Amresco, USA). The stain was removed, and the cells were washed twice with 60% isopropanol. About 1.5 mL of isopropanol was added to the stained 96-well culture plates for 5 min after washing with PBS, and the plate was agitated for 5 min on a shaker to dissolve formazan. The 550 nm absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Analysis of Caspase-3 activity

The activity of Caspase-3, a central component of the proteolytic cascade during apoptosis, was assessed in cell lysates using a Caspase-3 colorimetric kit (Assay Designs, Bologna, Italy). Briefly, preadipocytes were scraped from the plates at 24 h after obestatin (0, 0.1, 1, 10 and 100 nM) addition, and the medium with the preadipocytes was centrifugated at 1500 rpm,