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Research paper

Identification and expression patterns of adipokine genes during adipocyte differentiation in the Tibetan goat (*Capra hircus*)

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A B S T R A C T

Adipokines are secreted by adipose tissue and play an important role in the regulation of lipid metabolism. However, the information regarding adipokines in goats is limited. *PPARγ* is a key gene in adipocyte differentiation and activates adipokine genes. Rosiglitazone is a *PPARγ* agonist and can promote the expression of *PPARγ* to increase the expression of lipogenesis-related genes. Therefore, investigation of the relationship between rosiglitazone and adipokines will help us to better understand the function of *PPARγ* in lipid metabolism in Tibetan goats. In this study, we cloned the resistin (*RETN*), apelin (*APLN*), fibroblast growth factor 21 (*FGF21*), and visfatin (*NAMPT*) genes from non-pregnant female Tibetan goat adipose tissue. *APLN* and *NAMPT* were predominantly expressed in the kidney, and *FGF21* was expressed at the highest levels in the liver in vivo. In fat tissues, the highest expression levels of *FGF21* and *RETN* were detected in omental fat, whereas their expression in perirenal and subcutaneous fat was extremely weak. *APLN* and *NAMPT* were abundantly expressed in omental and subcutaneous fat in vivo. In addition, the four adipokines had different expression profiles during goat adipocyte differentiation in vitro. Oil red O staining showed that rosiglitazone could promote adipocyte differentiation and lipid droplet formation. In addition, rosiglitazone significantly increased the expression of *FGF21* and *RETN* ($p < 0.05$) but decreased the expression of *APLN* and *NAMPT* ($p < 0.05$). These results suggest that the four adipocytokine genes may have different roles during goat adipocyte differentiation. And PPARγ could regulate the expression of the four adipokines, but the detailed regulatory mechanism still needs to be elucidated.

1. Introduction

The function of adipose tissue has long been considered fat storage. Further study of adipose tissue, however, revealed that it can also produce and secrete fat factors called adipokines (Von, 1906). Adipokines play an important role in regulating metabolism, which controls fat accumulation, energy expenditure and glycometabolism (Cancello et al., 2004; Fasshauer and Blüher, 2015; Eichelmann et al., 2017). Leptin was the first adipokine to be discovered (Zhang et al., 1994); other adipokines, such as adiponectin (Maeda et al., 2012), resistin (RETN) (Steppan et al., 2001), apelin (APLN) (Boucher et al., 2005), fibroblast growth factor 21 (FGF21) (Nishimura et al., 2000), and visfatin (NAMPT) (Fukuhara et al., 2005), were found in subsequent studies. Adipokines travel through the endocrine or paracrine system to act upon other tissues, and research on FGF21 and NAMPT includes typical examples of functional studies of adipokines (Fukuhara et al., 2005; Kusminski et al., 2005; Lee et al., 2006; Markan et al., 2014). FGF21 is mainly produced in the liver and is then secreted to act upon adipose tissue and other tissues in mouse (Nishimura et al., 2000). Intracellular NAMPT is a key enzyme in the synthesis of nicotinamide adenine dinucleotide (NAD) (Jacques et al., 2012). Extracellular NAMPT increases glucose uptake and insulin sensitivity (Revollo et al., 2007). Similarly, in mammals, NAMPT is produced abundantly by adipose tissue to increase the amount of visceral fat in mice and humans, but it is also secreted by muscle, liver and other tissues (Fukuhara et al., 2005). Recent studies have elucidated that NAMPT plays the role of a myokine to regulate energy metabolism and is extensively expressed in skeletal muscle in chickens (Krzysik-Walker et al., 2008), rat (Wang et al., 2010) and mice (Maillard et al., 2017). In addition, RETN was originally detected in adipose tissue in mice and is also distributed in skeletal

Abbreviations: FGF21, fibroblast growth factor 21; APLN, apelin; RETN, resistin; NAMPT, visfatin; RELM, resistin-like molecule; NAD, nicotinamide adenine dinucleotide; ORO, Oil red O; TG, triglyceride; PPARγ, peroxisome proliferator-activated receptor γ

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muscle, spleen and other tissues (Steppan et al., 2001; Kusminski et al., 2005). Similarly, in humans, the expression of APLN is highest in lung tissue but is also present in brain, liver, and other tissues (Medhurst et al., 2003).

Currently, only basic functional information on adipokines is available. Leptin plays an important role in regulating energy metabolism and maintaining body weight; also, it increases glycolysis and inhibits hepatic gluconeogenesis (Michel et al., 2016). Adiponectin mediates glucose homeostasis and insulin sensitivity in murine tissues (Lin et al., 2013). RETN belongs to the RETN-like molecule (RELM) hormone family. It affects lipid metabolism during adipocyte maturation (Ikeda et al., 2013). It also plays a critical role in maintaining fasting glycemia levels and in the formation of excess white adipose tissue (Steppan et al., 2001). The expression of RETN is obviously inhibited by high insulin levels in 3T3-L1 cells (Haugen et al., 2001). In adipose tissue, the accumulation of fat cells can increase the expression of *APLN* (Boucher et al., 2005). *APLN* mRNA levels increase during the differentiation of 3T3-L1 cells to promote the maturation of adipocytes (Wei et al., 2005). APLN plays a role in increasing the insulin sensitivity of adipose tissues (Boucher et al., 2005). In addition, APLN can also improve glucose metabolism and increase insulin resistance (Dray et al., 2008). NAMPT upregulates the tyrosine phosphorylation of IRS-1 protein and induces the activation of both insulin receptor and ERK1/2 to improves insulin sensitivity (Sun et al., 2009; Brown et al., 2010). FGF21 reduces body weight, increases energy expenditure, and improves insulin sensitivity (Xu et al., 2009; Chau et al., 2010).

Adipokine genes are associated with adaptation to high altitude. FGF21 could be involved in bodily functions under simulated high-altitude conditions via the AMPK signaling pathway (Zhou et al., 2016). APLN promotes the oxygenation of hemoglobin under hypobaric hypoxia (Mishra et al., 2015). RETN regulates endothelial dysfunction among young Tibetan male adults, and it contributes to glucose tolerance in hypoxic conditions at high-altitudes (Al Mutairi et al., 2011; Yang et al., 2012). NAMPT is significantly decreased in human skeletal muscle at high altitudes (Acs et al., 2014). The Tibetan goat is distributed mainly in the Qinghai-Tibet plateau area and is characterized as having crude feed tolerance, high disease resistance and good adaptability but slow growth. The four adipokine genes discussed above may be related not only to the metabolism but also to the adaptation of the Tibetan goat to high altitude.

Adipokines are involved in the differentiation and maturation of adipocytes. The expression of FGF21 promotes lipid accumulation and adipocyte differentiation in human abdominal subcutaneous adipocytes (Berti et al., 2015). APLN suppresses adipogenesis and lipolysis during the differentiation of 3T3-L1 cells (Than et al., 2012). The expression of NAMPT regulates lipogenesis in porcine adipocytes by activating the transcription of fatty acid synthase and stimulating adipocyte differentiation (Yang et al., 2010). RETN modulates adipogenesis by regulating the phosphorylation of different kinases involved in adipogenesis in 3T3-L1 preadipocytes (Sanchez-Solana et al., 2012). In this study, the four adipokine genes were cloned and their mRNA expression patterns were detected in different tissues of the Tibetan goat. In addition, we also studied the expression of these four adipokine genes during preadipocyte differentiation in vitro. Previous studies have demonstrated that PPARγ can regulate the expression of adipokine genes and is also a key gene in adipocyte differentiation (Lehrke and Lazar, 2005; Siersbaek et al., 2010). Rosiglitazone is a PPARγ agonist, and it can promote the expression of PPARγ to increase the expression of lipogenesis-related genes (Milton et al., 2017). Therefore, investigation of the relationship between rosiglitazone and adipokines will help us to better understand the function of PPARγ in lipid metabolism in Tibetan goats.

2. Materials and methods

2.1. Ethics statement

All research involving animals was conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004) and approved by the Institutional Animal Care and Use Committee at the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China, under permit No. DKY-B20110807.

2.2. Animals and sample collection

Six non-pregnant female Tibetan goats (2 years old) from Aba Prefecture (high-altitude area), Sichuan Province, were used to examine the levels of tissue expression. Eleven types of tissue (heart, liver, spleen, lung, kidney, brain, *longissimus dorsi* muscle, *semitendinosus* muscle, perirenal fat, omental fat and subcutaneous adipose) were collected in the morning within 30 min after slaughter and immediately frozen in liquid nitrogen. In addition, three female Tibetan goats (3 days old) were used to isolate preadipocytes. Samples from each goat were collected in the cell culture room after death by carotid artery bleeding. The subcutaneous adipose tissues were collected from the back of the goats. Then, the tissues were washed with PBS containing 5% antibiotics, and blood vessels and muscles were removed from the tissue surfaces.

2.3. Cloning of the goat FGF21, APLN, RETN, and NAMPT genes

Primer pairs (Table 1) were designed to amplify the goat genes according to regions of conservation compared to other species (cattle, human and mouse) using Primer Premier 6.0 software. First, we determined the Tm of the primers by gradient PCR. Then, we evaluated each gene by PCR. The total volume of the PCR reaction mixture was 30 μL, which contained 15 μL of mix, 1 μL each of the forward and reverse primers, 11.5 μL of ddH_2O, and 1.5 μL of normalized template cDNA. The PCR products were separated by electrophoresis on 2.0% agarose gels and purified using a Gel Extraction Kit (Omega). The purified products were subcloned into the pMD-19T vector (Takara, Japan) and sequenced by Sangon Biotechnology Company (Shanghai, China).

2.4. Isolation and differentiation of goat preadipocytes

Under aseptic conditions, 3 g of subcutaneous adipose tissue was removed from three female Tibetan goats (3 days old) and washed three times with PBS. The adipose tissue was cut into 1-mm^3 pieces and digested for 1 h (37 °C, mixed every 5 min) in 1% type I-collagen enzyme. The digested sample was filtered through 70-μm and 40-μm double-layer nylon sieves. The filtrate was centrifuged for 10 min at 1000 × *g*, and the supernatant was discarded. The pellet was suspended in red blood cell lysis buffer (Beyotime, Haimen, China), and then incubated at room temperature for 5 min. The cell suspension was centrifuged for 5 min at 1000 × *g*, and the supernatant was discarded. The pellet was suspended in PBS and then centrifuged for 5 min at 1000 × *g*; the supernatant was discarded. The pellet was washed twice with complete medium (15% fetal bovine serum (FBS) + 2% antibiotics (200 U/mL penicillin and 200 μg/mL streptomycin) + DMEM/F12) to obtain a suspension solution. The cell suspension solution was transferred to a culture bottle and cultured in complete medium at 37 °C. The media were replaced after 24 h to remove impurities and dead cells. Then, the primary adipocytes from each lamb were seeded in three wells to study transcription levels.

The preadipocytes were cultured in DMEM/F12 (HyClone, Logan, UT) medium supplemented with 10% FBS and 2% antibiotics (growth

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