Lipolysis and apoptosis of adipocytes induced by neuropeptide Y-Y5 receptor antisense oligodeoxynucleotides in obese rats[1]

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ABSTRACT

AIM: To investigate the influence of central administration of neuropeptide Y-Y5 receptor antisense oligodeoxynucleotides (ODN) on the body weight and fat pads of high-energy diet-induced obese rats, and the effects on white adipocyte lipolysis and apoptosis. **METHODS:** Y5 receptor antisense, sense, mismatched oligodeoxynucleotides (ODN) or vehicle were intracerebroventricularly injected, and average adipocyte area was calculated. DNA ladders were measured to evaluate adipocyte apoptosis, and RT-PCR was used to analyze the expression of *bcl-2* and *bax* gene. **RESULTS:** (1) Central administration of Y5 receptor antisense ODN significantly decreased body weight, fat pads, and average adipocyte area. (2) DNA fragmentation was presented after electrophoresis at both epididymal and retroperitoneal adipose tissue. (3) The expression of *bcl-2* gene was downregulated, while the expression of *bax* was upregulated. **CONCLUSION:** Lipolysis and adipocyte apoptosis may be important reasons for Y5 receptor antisense therapy.

INTRODUCTION

The disarrangement of appetite regulation is tightly associated with obesity,a serious health problem in modern society[1]. Neuropeptide Y (NPY), an important determinant in obesity onset and the most powerful orexigenic agent discovered to date, appears to play a central role in the control of feeding behavior[1,2]. NPY exerts its physiological effects by activating a family of G-protein coupled receptors, among which Y1 and Y5 receptor are intimately involved in the regulation of appetite[3]. But *in vivo* and *in vitro* characterization of the recently-cloned Y5 receptor subtype suggests that it may be a primary mediator of NPY-induced feeding[4-6]. Central administration of Y5 receptor antisense oligodeoxynucleotides (ODN) decreases spontaneous and fasting-induced food intake and induces significant body-weight loss[5,6]. Intraperitoneal injection of the highly selective Y5 receptor antagonist CGP71683A dramatically reduces body weight and peripheral fat mass[4]. Whereas adverse conclusions are also achieved

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from Flynn and coworkers, who found that Y5 receptor antisense ODN had no effect on either spontaneous food intake or NPY, NPY analogue stimulated food intake[7]. So the importance of Y5 receptor among five NPY receptor subtypes in mediating feeding response and the mechanisms for body weight mitigating induced by NPY-Y5 receptor axis blockage have not been clearly elucidated.

Excessive adipose tissue deposition is attributed to an imbalance between energy intake and energy expenditure, which is accompanied by an increase in adipocyte size and/or number[8]. Adversely, dramatic body weight loss is often accompanied by white adipocyte apoptosis or lipolysis. So we hypothesized that body weight loss induced by functional blockage of NPY-Y5 axis might be the results of adipocyte apoptosis and lipolysis. In the absence of specific Y5 antagonists or Y5 receptor knockout mice, ODN are used to study the function of Y5 receptor *in vivo*. In this context, phosphothioate end-protected antisense ODN targeted to the initiation codon of Y5 receptor mRNA was intracerebroventricularly injected.

One important molecular mechanism for cell apoptosis might be the modulation of the expression of *bcl-2* gene family, which contains both antiapoptotic (such as *bcl-2*) and proapoptotic (such as *bax*) members[9]. The mRNA and protein ratio of *bcl-2* to *bax* is a pivotal factor to determine brown adipocyte apoptosis. But their expression and action in white adipocytes have not been reported. So in our study, the expression of *bcl-2* and *bax* gene in white adipose tissue was also detected.

MATERIALS AND METHODS

Animals and diet Four-eight weanling male SD rats, supplied by Animal Center of Jiangsu Province, were housed at 21-23 °C with a 19:00-7:00 h dark cycle, and allowed a free access to food and water for at least a week before grouping. Then these rats were fed with a high-energy diet to generate an obesity model[10]. High-energy diet components were milk powder 10 g, lard 10 g, concentrated fish liver oil 10 drops, fresh bean sprout 250 g, and an egg per 100 g chow, which were given to each rat daily as published elsewhere[9]. Animals received this diet for 7 weeks. All of the animal experiments were conducted in accordance with the highest standards of human care.

Surgery[6] Under sodium pentobarbitol (50 mg/kg) anesthesia, forty-eight obese rats were stereotaxically implanted with stainless steel guide cannulas extending 3.75 mm below the exernal surface of the skull. The cannula was placed into the anterior horn of the lateral ventricle through a trepanation located 1 mm lateral to the bregma. After cannulation, the rats were individually housed in metabolic cages, allowing precise measurements of food intake. The animals were allowed a 7-d recovery period during which they were handled daily to habituate them to the injection procedure.

ODN[6] Phosphothioate end-protected ODN were obtained from Shanghai BioAsia Bio Technology Co, Ltd. For the NPY-Y5 receptor ODN spanned 20 bp downstream from the start codon, a previous published sequence was used. Antisense ODN: 5'-AGA GGA CGT CCA TTA GCA GC-3', mismatched ODN: AGA GTA CGG CCA CTA GCA GT-3', sense ODN: GCT-GCT AAT GGA CGT CCT CT-3'.

Injection paradigm[6] Before experiments were started, rats were randomized into 4 groups. One group received the antisense ODN, and other groups received sense, mismatched ODN or vehicle respectively. The rats were injected thrice daily (10:00 AM, 1:00 PM, 4:00 PM) for 2 d with 50 OD in 10 μL saline each time. Food intake and body weight were monitored every 24 h during the following 2 d. On the third day (after a total six injections) rats were decapitated. Epididymal and retroperitoneal adipose tissues were precisely measured and stored under -70 °C. Trunk blood was also collected.

Total RNA isolation and amplification by RT-PCR Total cytoplasmic RNA was isolated from adipocyte tissue by the TRIZOL method (GIBCO specification). For PCR analysis, the RNA was treated at 37 °C for 1 h with 6 U ribonuclease (RNase)-free deoxyribonuclease I every 1 μg RNA. One microgram of total RNA was converted to complementary DNA (cDNA) using 200 U Moloney murine leukemia virus reverse transcriptase (Promega, USA) in 20 μL buffer, which contained 0.5 mmol/L deoxy-NTP, 20 U RNase inhibitor, and 0.1 μg oligo(deoxythymidine)$_{15}$ primer (Promega). An aliquot (5 %) of the cDNA was amplified using the reverse (5'-AGA GGG GCT ACG AGT GGG AT-3') and forward primer (5'-CTC AGT CAT CCA CAG GGC GA-3') to yield a 450 bp PCR product for *bcl-2* [9], the reverse (5'-GGT TTC ATC CAG GAT CGA GAC GG-3') and forward primer (5'-AGA AAG ATG GTC ACG GTC TGC c-3') to yield a 429 bp PCR product for *bax* [9], and the reverse (5'-TAA AGA CCT

CTA TGC CAA CAC AGT-3') and forward primer (5'-CAC GAT GGA GGG GCC GGA CTC ATC -3') to yield a 240 bp PCR product for β-actin[11]. The PCR conditions were as follows: denaturation at 94 °C for 40 s, annealing at 60.2 °C (*bcl-2* and β-actin) or 64 °C (*bax*) for 40 s and polymerization at 72 °C for 40 s with truncated *Thermus aquatious* DNA polymerase (Promega). The total cycles were 25 for *bcl-2* and β-actin or 35 for *bax*. Three gene fragments were amplified respectively. PCR products 10 μL were detected by electrophoresis and ethidium bromode staining.

The number of cycles for the semiquantitative RT-PCR assay and the reaction temperature conditions were optimized to provide a linear relationship between the amount of input template and the amount of PCR product generated over a wide concentration range: from 0.5 to 10 μg of total RNA.

DNA fragment analysis Adipocyte tissue samples (100 mg) were homogenized in 10 mmol/L Tris-HCl (pH 7.5), 0.32 mol/L sucrose, 5 mmol/L $MgCl_2$, 0.5 % lauryl sarcosyl containing 200 mg/L of proteinase K, and then incubated at 55 °C for 1 h. The DNA was subsequently precipitated overnight with ethanol, recovered by means of centrifugation in water. DNA 20 μL was loaded onto 1.5 % agarose gel, which was stained with ethidium bromide after migration.

Morphological evaluation of white adipocytes Serial slices of adipose tissue were prepared from rats in each group and stained with hematoxyline-eosin. Morphological evaluation was performed in five optical fields randomly taken in each slide at ×200 magnification using MPIAS (multimedia pathological image & word analysis system)-500 software.

Statistical analysis All results were expressed as mean±SD. Data were analyzed using one-way ANOVA and *t* or *t'* tests with a correction for multiple comparisons, as appropriate.

RESULTS

Spontaneous food intake Central administration of NPY-Y5 receptor antisense ODN decreased spontaneous food intake throughout the observation period, while mismatched ODN or vehicle had no effect on cumulated 48-h food intake. Unexpectedly, rats that received sense ODN therapy also decreased food intake (Tab 1).

Body weight Injection (icv) of Y5 receptor antisense ODN decreased body weight when compared with both vehicle and mismatched ODN (Tab 1). And in accord with food intake, Y5 receptor sense ODN treatment also significantly decreased body weight. However, this reduction is smaller than that by antisense ODN (Tab 1).

WAT mass and the average area of adipocyte As shown in Tab 2, Y5 receptor antisense and sense ODN induced a significant fall in the average area of adipocytes both at retroperitoneal and epididymal adipose tissue. But only the weight of the retroperitoneal fat pats was reduced in antisense group.

Adipocyte genomic DNA fragment analysis Y5 receptor antisense and sense therapy induced adipocyte apoptosis. In this regard, adipocyte genomic DNA was extracted and migrated on 1.5 % agarose gel, and DNA fragmentation was presented after electrophoresis at both epididymal and retroperitoneal WAT (Fig 1).

Detection of *bcl-2* and *bax* in WAT preparations In accord with the results mentioned above, NPY-Y5 receptor antisense or sense ODN therapy decreased *bcl-2* expression and increased *bax* expression both in

Tab 1. Effects of Y5 receptor antisense oligodeoxynucleotides (ODN) on spontaneous food intake and body weight in obese rats. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Vehicle.

	n	Wb/g	W1/g	W2/g	WR1/g	WR2/g	FIb/g	FI1/g	FI2/g
Vehicle	12	379±44	372±45	361±44	7±4	18±5	43±5	42±8	43±8
Mismatched	10	383±37	372±38	360±37	11±4	23±7	41±5	39±4	40±6
Antisense	9	380±39	344±33	316±30^b	36±15^c	64±29^c	41±8	23±6^c	25±4^c
Sense	7	379±43	362±46	349±47^b	17±6^c	30±9^c	39±5	34±4^b	31±3^b

W: body weight. WR: body weight reduction. Wb: body weight before treatment. W1: body weight after 1-d treatment. W2: body weight after 2-d treatment. FI1: food intake on d 1. FI2: food intake on d 2.

Tab 2. Effects of Y5 antisense ODN on peripheral fat pats and average adipocyte area in obese rats. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* Vehicle.

	n	We/g	Wr/g	Ae/μm^2	Ar/μm^2
Vehicle	12	5.4±2.2	7.2±3.2	1902±173	2577±342
Mismatched	10	4.7±1.7	5.2±2.2	1892±152	2514±239
Antisense	9	4.5±1.8	2.5±2.2^{c}	1522±94^{c}	1405±344^{b}
Sense	7	4.8±2.6	6.0±4.3	1708±137^{b}	1754±419^{c}

We: weight of fat pad at epididymal. Wr: weight of fat pad at retroperitoneal. Ae: average area of adipocyte at epididymal. Ar: average area of adipocyte at retroperitoneal.

epididymal and retroperitoneal WAT (Fig 2). The ration of *bcl-2* to *bax* was decreased.

DISCUSSION

Obese patients often have augmented appetite, which either temporary or permanent, invariably culminates in an increased rate of body weight gain and obesity[1]. There is now a growing recognition that appetite is chemically regulated by several kinds of neurotransmitter in the hypothalamus. NPY is the most powerful orexigenic neurotransmitter discovered to date[1].

The actions of NPY are to be mediated by several receptor subtypes named NPY Y1-Y6[12]. Recent studies correlating the Y5 binding affinity of a range of NPY peptide agonists with their ability to induce food intake suggest that this receptor is intimately involved in NPY-induced feeding[13,14]. But adverse conclusions were also achieved from Flynn and coworkers[7]. Our study, together with recently published studies by Schaffhauser and Tang-Christensen, provides evidence that central Y5 receptor is important mediator of the orexigenic properties of NPY. We have demonstrated that six doses of Y5 receptor antisense ODN significantly reduced spontaneous food intake and body weight in *ad libitum* obese rats. These reductions were more pronounced in the first days. Although there was no further reduction in food intake, body weight loss was accompanied by reduction in fat mass especially in retroperitoneal adipose tissue. These indicate that other neuronal systems whose functions included in control food intake are rapidly recruited to compensate for the loss of NPY activity[15]. In the brain, NPY has two effects on energy metabolism in addition to increasing feeding, namely, decreasing brown fat thermogenesis and increasing white fat lipoprotein lipase activity, both of which are secondary to increased central sympathetic outflow[16]. So the loss of body weight and fat mass is due to an increase in central sympathetic outflow and a decrease in food intake. However, injection of sense ODN also significantly decreased food intake and body weight. The possible reason is that the sense ODN of Y5 receptor have a high homology to antisense sequences of coding regions of a number of constitutively expressed neural tissue proteins[6].

WAT is the main location where individuals store

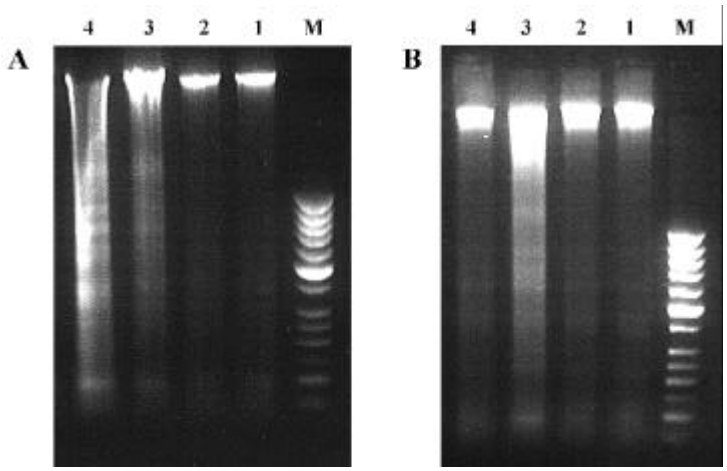

Fig 1. NPY-Y5 receptor antisense and sense ODN induced ladder pattern fragmentation of the genomic DNA at epididymal (A) and retroperitoneal white adipose tissue (WAT) (B), a hallmark of apoptosis. M: molecular weight markers, 1: saline-treated WAT; 2: mismatched ODN-treated WAT; 3: sense ODN-treated WAT; 4: antisense ODN-treated WAT.

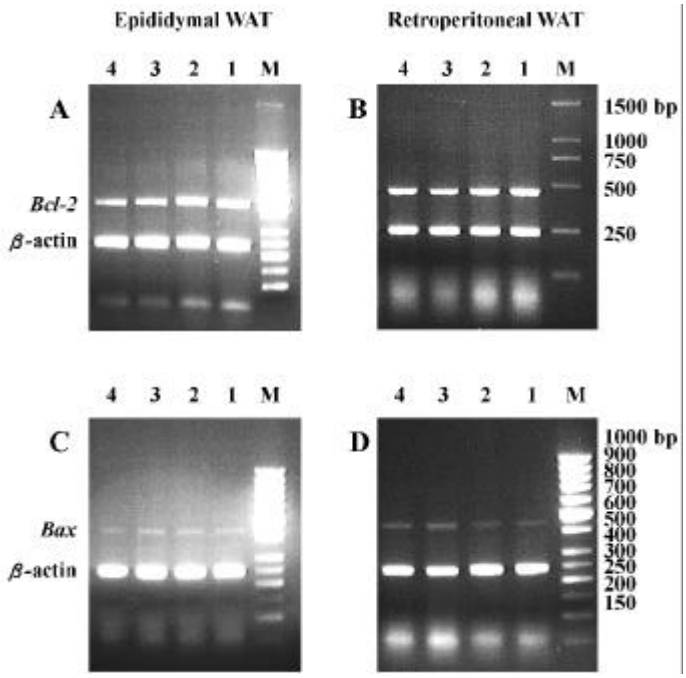

Fig 2. Analysis of the *bcl-2* and *bax* mRNA expression in WAT. *bcl-2* (A, B) and *bax* (C, D) fragments amplified by RT-PCR from various preparations were electrophoresised in ethidium bromide stained in 2 % agarose gel. M: molecular weight markers (bp). 1: saline-treated WAT; 2: mismatched ODN-treated WAT; 3: antisense ODN-treated WAT; 4: sense ODN-treated WAT.

energy. In different conditions of energy balance, individuals modulate energy efficiency and nutrient partitioning between adipose tissue and other tissues[17,18]. In the positive energy balance, selective fatty acid uptake and lipogenesis were up-regulated by adipocyte. Excessive energy present in the form of fatty acid flows to adipocyte and stores in the form of triglyceride[19]. So the weight of WAT and average adipocyte area increase. On the contrary, when lipolysis in WAT and oxidation of fatty acid increase, the weight of WAT and average adipocyte area decrease. In our experiments, energy metabolism was present in a negative balance after antisense therapy. It is not surprising that fat mass and average adipocyte area were reduced. In addition, we found that the change of fat mass at retroperitoneal adipose tissue was more obvious than that at epididymal adipose tissue. This may indicate that retroperitoneal WAT was the main target tissue responsible for Y5 antisense therapy. But the mechanisms have not yet been elucidated. We thought that it was caused by different sensitivity to the regulation of gene expression associated with lipid metabolism in adipocytes[20].

Furthermore, the number of adipocytes is also an important determinant for fat mass[21]. If the differentiation of preadipocytes increase and adipocyte apoptosis decrease, adipose tissue accumulation will occur. Adverse to the process mentioned above, slimming effects would be reached. It is definitive that adipocyte apoptosis was tightly related to weight loss[22,23]. Our results of oligonucleosome-sized fragments of nuclear DNA in electrophoresis suggest that adipocyte apoptosis did occured after Y5 receptor antisense therapy.

It was reported that in SD rats exposed to low temperature for 3 d, the *bcl-2*/*bax* ratio was markedly increased at BAT, and brown adipocyte was prevented from apoptosis[8]. To understand the molecular mechanisms involved in Y5 receptor antisense therapy, the expression of *bcl-2* and *bax* in WAT was also investigated. Given that *bcl-2* is a cell-death repressor and *bax* a cell-death promoter, the ratio between them is a determinant for cell fate[9]. We found that *bcl-2* and *bax* mRNA were presented in white adipocyte. Furthermore, the gene expression was makedly modified in antisense ODN-treated rats with decreased *bcl-2* and increased *bax* mRNA levels. This change is compatible with increased adipocyte apoptotic death after Y5 receptor antisense ODN treatment. These results further support the possibility that white adipocyte apoptosis may contribute to the development of Y5 receptor antisense therapy-linked body weight loss.

It seems contradictory that adipocyte apoptosis increased and average adipocyte area decreased after antisense administration, but the difference of fat mass among four groups did not reach statistical significance. Qian and coworkers demonstrated that injection of leptin could dramatically up-regulate the expression of peroxisome proliferator-activated receptor γ (PPARγ), which induced adipocyte apoptosis[23]. Meanwhile, PPARγ is a powerful promotor for preadipocyte differentiation[20]. So the minor change of the WAT weight at epididymal may be the synthetic results of increased number of small adipocyte undergone proliferation and increased number of big adipocyte undergone apoptosis. This hypothesis is in accord with the report that troglitazone increased the number of small adipocyte without change of white adipose tissue mass in obese zucker rats[24].

In summary, our study raises the possibility that the NPY-Y5 receptor antisense therapy may delete WAT by inducing adipocyte apoptosis and lipolysis. Therefore Y5 receptor antagonistic treatment is a promising new method for obesity therapy. Other methods that can be used to induce white adipocyte apoptosis or lipolysis may also be useful to obese treatment.

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