รายงานวิจัยฉบับสมบูรณ์

โครงการ การแสดงออกของจีนและโปรตีน ของตัวรับชนิดที่ 1, 2 และ 5 ของ นิวโรเปปไทด์วายในเนื้อเยื่อไขมันในคนผอม ปานกลาง และอ้วน และความสัมพันธ์ต่อดัชนีมวลกายและระดับสารเมตาบอลิกในเลือด

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มีนาคม พ.ศ. 2556

สัญญาเลขที่ MRG5380013

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

เอกสารแนบหมายเลข 2

บทคัดย่อ

รหัสโครงการ: MRG5380013

ชื่อโครงการ: โครงการ การแสดงออกของจีนและโปรตีน ของตัวรับชนิดที่ 1, 2 และ 5 ของนิวโรเปปไทด์วายในเนื้อเยื่อไขมันในคนผอม ปานกลาง และอ้วน และความสัมพันธ์ต่อดัชนีมวลกายและระดับสารเมตาบอลิกในเลือด

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ระยะเวลาโครงการ: 3 ปี ตั้งแต่ 15 มิถุนายน 2553 ถึง 14 มิถุนายน 2556

นิวโรเปปไทด์วายพบในระบบประสาทส่วนกลางโดยเฉพาะสมองส่วนไฮโพทาลามัสซึ่งมีบทบาทสำคัญในการควบคุมความอยากอาหาร การศึกษาก่อนหน้านี้พบว่ามีการแสดงออกของจีนตัวรับของนิวโรเปปไทด์วายชนิดที่ 1, 2, และ 5 ในเนื้อเยื่อไขมัน การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบการแสดงออกของจีนตัวรับของนิวโรเปปไทด์วายชนิดที่ 1, 2, และ 5 ในเนื้อเยื่อไขมันหน้าท้องและเนื้อเยื่อไขมันของอวัยวะในช่องท้องระหว่างคนน้ำหนักปกติและคนอ้วน นอกจากนี้ยังศึกษาหาความสัมพันธ์ของการแสดงออกของจีนต่างๆ กับระดับสารแมแทบอลิกในเลือดและข้อมูลทางคลินิก การศึกษานี้พบว่าตัวรับของนิวโรเปปไทด์ชนิดที่ 1 และชนิดที่ 5 สูงในคนอ้วนเทียบกับคนน้ำหนักปกติในเนื้อเยื่อไขมันของอวัยวะในช่องท้อง (ค่านัยสำคัญทางสถิติ < 0.05) และตัวรับของนิวโรเปปไทด์ชนิดที่ 5 สูงในคนอ้วนเทียบกับคนน้ำหนักปกติในเนื้อเยื่อไขมันหน้าท้อง (ค่านัยสำคัญทางสถิติ < 0.05) ในทางตรงกันข้ามการแสดงออกของจีนตัวรับของนิวโรเปปไทด์วายชนิดที่ 2 ในเนื้อเยื่อไขมันของอวัยวะในช่องท้องสูงในคนน้ำหนักปกติเทียบกับคนอ้วน (ค่านัยสำคัญทางสถิติ<0.05) เมื่อเปรียบเทียบระหว่างเนื้อเยื่อไขมันหน้าท้องและเนื้อเยื่อไขมันของอวัยวะในช่องท้อง พบว่า การแสดงออกของจีนตัวรับนิวโรเปปไทด์วายชนิดที่ 1 สูงในเนื้อเยื่อไขมันหน้าท้องในคนอ้วนและอาสาสมัครทั้งหมด (ค่านัยสำคัญทาง

สถิติ < 0.05) แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติในคนน้ำหนักปกติ การแสดงออกของจีนตัวรับนิวโรเปปไทด์วายชนิดที่ 2 สูงในเนื้อเยื่อไขมันหน้าท้องในคนอ้วน (ค่านัยสำคัญทางสถิติ < 0.05) แต่ไม่แตกต่างกันในคนน้ำหนักปกติและอาสาสมัครทั้งหมด ตัวรับนิวโรเปปไทด์วายชนิดที่ 5 ไม่มีความแตกต่างระหว่างเนื้อเยื่อไขมันหน้าท้องและเนื้อเยื่อไขมันของอวัยวะในช่องท้อง ในคนอ้วน คนน้ำหนักปกติและอาสาสมัครทั้งหมด ในคนอ้วนพบการเพิ่มขึ้นอย่างมีนัยสำคัญของความดันเลือดขณะหัวใจบีบตัว (ค่านัยสำคัญทางสถิติ < 0.01) ระดับความดันเลือดขณะหัวใจคลายตัว (ค่านัยสำคัญทางสถิติ < 0.05) ระดับสารอินซูลินในเลือด (ค่านัยสำคัญทางสถิติ < 0.05) ดัชนีชี้วัดภาวะดื้อต่ออินซูลิน (ค่านัยสำคัญทางสถิติ < 0.05) นอกจากนี้การแสดงออกของจีนตัวรับของนิวโรเปปไทด์วายชนิดที่ 1 และ 5 ในเนื้อเยื่อไขมันของอวัยวะในช่องท้องมีความสัมพันธ์เชิงบวกกันอย่างสูง (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.773) การแสดงออกของจีนตัวรับของนิวโรเปปไทด์วายชนิดที่ 1 ในเนื้อเยื่อไขมันของอวัยวะในช่องท้องมีความสัมพันธ์เชิงบวกกับน้ำหนักตัว (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.586) ดัชนีมวลกาย (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.611) รอบเอว (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.474) รอบสะโพก (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.483) ระดับสารอินซูลินในเลือด (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.539) และดัชนีชี้วัดภาวะดื้อต่ออินซูลิน (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.480) การแสดงออกของตัวรับนิวโรเปปไทด์วายชนิดที่ 5 ในเนื้อเยื่อไขมันของอวัยวะในช่องท้องมีความสัมพันธ์เชิงบวกกับน้ำหนักตัว (ค่าสัมประสิทธิ์สหสัมพันธ์= 0.415) ดัชนีมวลกาย (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.453) รอบสะโพก (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.418) และระดับสารเลปตินในเลือด (ค่าสัมประสิทธิ์สหสัมพันธ์ = 0.435) โดยสรุปแล้วการแสดงออกของจีนตัวรับนิวโรเปปไทด์วายชนิดที่ 1 และ 5 มีความสัมพันธ์เชิงบวกกับความอ้วนและปัจจัยเสี่ยงของโรคแมแทบอลิก การศึกษาในอนาคตเกี่ยวกับการยับยั้งหรือกระตุ้นการออกฤทธิ์ของตัวรับนิวโรเปปไทด์วายชนิดที่ 1, 2, และ 5 ในเนื้อเยื่อไขมัน อาจจะนำมาใช้เป็นกลยุทธ์ใหม่สำหรับการป้องกันหรือการรักษาความอ้วน

คำหลัก: ตัวรับนิวโรเปปไทด์วายชนิดที่ 1, ตัวรับนิวโรเปปไทด์วายชนิดที่ 2, ตัวรับนิวโรเปปไทด์วายชนิดที่ 5, เนื้อเยื่อไขมัน, อ้วน

Abstract

Project Code: MRG5380013

Project Title: Expression of mRNA and protein of NPY receptors, Y1, Y2, Y5 in subcutaneous and visceral fat tissues in lean, normal, and obese human and their correlations to peripheral metabolic factors

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Project Period: 3 years started 15 June 2010 to 14 June 2013

Neuropeptide Y (NPY) is expressed in the brain, especially in the hypothalamus, and plays a central role in appetite regulation. Recently, Y1 receptor (Y1R), Y2 receptor (Y2R), and Y5 receptor (Y5R) were found to be expressed in adipose tissue. This study aimed to compare Y1R, Y2R, and Y5R mRNA in subcutaneous and visceral fat tissues between normal weight and obese humans. Correlations between gene expressions, clinical parameters, and peripheral metabolic factors were also determined. We demonstrated that Y5R mRNA expressions were higher ($p<0.05$) in obese than in normal weight humans in both subcutaneous and visceral fat and Y1R in visceral fat was greater ($p<0.01$) in the obese group. On the contrary, Y2R mRNA in visceral fat was higher ($p<0.05$) in normal weight subjects than in obese subjects. Y1R mRNA was lower ($p<0.05$) in visceral fat when compared to subcutaneous fat in obese, normal weight, and overall subjects without significant difference for Y1R in normal weight subjects. Y2R mRNA was also more highly ($p<0.05$) expressed in subcutaneous than visceral fat tissue in obese subjects. There was similar expression between subcutaneous and visceral fat for Y2R mRNA in normal weight and overall subjects as

well as for Y5R mRNA in obese, normal weight, and overall subjects. Obese subjects showed significantly greater systolic blood pressure (SBP) ($p<0.01$), diastolic blood pressure (DBP) ($p<0.05$), plasma insulin ($p<0.05$), HOMA-IR ($p<0.05$), as well as serum NPY levels ($p<0.05$) than normal weight subjects. Additionally, there was a strong positive correlation between Y1R and Y5R mRNA ($R=0.773$) in visceral fat. Y1R mRNA in visceral adipose tissue was positively correlated with body weight ($R=0.586$), BMI ($R=0.611$), waist ($R=0.474$) and hip ($R=0.483$) circumferences, insulin levels ($R=0.539$), and HOMA-IR (0.480). The Y5R gene in visceral adipose tissue had positive correlations with body weight ($R=0.415$), BMI ($R=0.453$), hip circumference ($R=0.418$), and serum leptin ($R=0.435$). As a result, Y1R and Y5R expressions in visceral adipose tissue were positively associated with obesity and risks of metabolic syndrome. Further studies regarding blocking or activating specific receptors which are Y1R, Y2R, and Y5R in adipose tissue may propose new strategies for prevention or treatment of adiposity.

Keywords: Y1R, Y2R, Y5R, adipose tissue, obesity

Executive Summary

Obesity is defined as abnormal or excessive fat accumulation causing many adverse health consequences (1, 2) and has become a public health problem worldwide with 475 million adults and up to 50 million children being obese (data for 2010) (3). For BMI classification of Asian population, BMI more than 25 are identified as obese, 23-24.9 as overweight, 18.5-22.9 as normal range and less than 18.5 as underweight (4). The prevalence of obesity in Thai adults population was nearly increased twice in the past two decades with rapid expansion from 18.2% in 1991 to 28.1% in 2004 (5). Obesity is strongly related with hyperinsulinemia (6, 7) and metabolic disorders (8) with high Homeostatic Model for Assessment of Insulin Resistance (HOMA-IR), systolic blood pressure (SBP), diastolic blood pressure (DBP) (9, 10), abdominal adiposity, dyslipidemia, hyperglycemia predisposing to type II diabetes, cardiovascular diseases, hypertension, degenerative arthritis, and psychosocial impairment (2, 11, 12).

Currently, analysis of gene expression in adipose tissue linking to adiposity has been interested and extensively studied. One important candidate was Neuropeptide Y (NPY) (13-16) which is the peptide containing 36 amino acids. NPY was discovered in the central nervous system (CNS) (17-20), especially in the arcuate nucleus (ARC) of the hypothalamus (17-24) and plays a central role in appetite regulation (25, 26). In peripheral tissue, NPY mRNA was detected in adrenal gland, adrenal chromaffin cells (27), sympathetic ganglia, tissues receiving dense sympathetic innervations (28-32), endothelial cells (33, 34) as well as adipose tissues including visceral adipose tissue of rats (16), subcutaneous adipose tissue of mice (15), and visceral and subcutaneous adipose tissues of humans (13). NPY protein was found in human adipocytes, abdominal subcutaneous, omental, and thigh adipose tissues and the expression in abdominal subcutaneous adipose tissue was doubled in omental or thigh adipose tissues (14).

NPY acts through a family of G-protein coupled receptors (35) which are Y1-Y7 receptors (36, 37) with neuropeptide Y1 receptor (Y1R), neuropeptide Y2 receptor (Y2R), neuropeptide Y4 receptor (Y4R), and neuropeptide Y5 receptor (Y5R) being presented in humans (35). Y1R, Y2R, and Y5R are abundantly expressed in the brain of rats (38, 39), mice (35, 40) and humans (24, 41) playing a major role in feeding regulation and are also found to be expressed in adipose tissue (15, 41, 42).

Y1R was identified centrally in the brain including the hypothalamus of rats (24) and humans (24, 41) and peripherally in adipocyte of rats (16), as well as uterus, skin, and subcutaneous adipose tissue of humans (41). The central effect of Y1R was involved in increased appetite (42, 43) whereas potential peripheral functions of included vasoconstriction (35) and proliferation of primary cultures of rat preadipocytes (16). Administration of Y1R antagonist reduced body weight and food intake in mice (42). NPY induced proliferation of rat preadipocytes as well as murine 3T3-L1 preadipocytes cell via Y1R acting through extracellular related kinase 1/2 (ERK1/2) (16). The strong antilipolytic effect of NPY was blocked by specific Y1R antagonists (44) enhancing the antilipolytic effect of NPY acting through Y1R.

Y2R was located in several brain areas (24, 39) and also in peripheral tissues such as preadipocytes and subcutaneous abdominal adipose tissue of mice (15). The main functions of Y2R were involved in the induction of satiety (35), promoting the proliferation and differentiation of adipocytes as well as stimulating angiogenesis of capillaries in adipose tissue of mice (15). Oral administration of Y2R agonist reduced body weight and cumulative food intake in diet-induced obese (DIO) (45). Moreover, Y2R mRNA was noticeably up-regulated in subcutaneous abdominal fat of obese leptin-deficient (ob/ob) mice compared with controls (15).

Y5R mRNA and protein were detected centrally in the hypothalamus (38, 40, 41) and peripherally in many tissues such as adrenal gland, vascular smooth muscle cells, and cardiomyocytes in rodent and human (24, 41). Recent study showed that Y5R mRNA was expressed in human adipocytes and subcutaneous adipose tissue (41). Previous study in mice demonstrated that Y5R antagonist suppressed spontaneous food intake (42); furthermore, the study showed synergistic interaction between the Y1R and Y5R in increasing food intake and body weight (42).

Collectively, NPY, Y1R, Y2R, and Y5R mRNA were detected centrally in the hypothalamus and peripherally in many tissues including adipose tissues (13-16, 41). NPY, Y1R, Y2R, and Y5R are associated with appetite regulation and adiposity with different roles and actions. The expressions might be differently detected in different levels of obesity as well as different type of adipose tissue. Revelation of information regarding different NPY receptor subtype expressions in various levels of adiposity and correlations between the expressions with clinical data and peripheral metabolic factors will elucidate the potential of specific receptors involved in obesity. Manipulation of specific NPY receptors differently expressed in

varieties of levels of adiposity such as blocking or activating specific receptor subtypes may propose new strategies for prevention and treatment of obesity.

Research questions

1. Whether Y1R, Y2R, and Y5R mRNA expressions are different between obese and normal weight subjects as well as between visceral and subcutaneous adipose tissues?

2. Are Y1R, Y2R, and Y5R mRNA and serum NPY levels correlated with clinical data and peripheral metabolic factors?

Hypotheses

1. Y1R, Y2R, and Y5R mRNA expressions in subcutaneous and visceral adipose tissues are different in obese and normal weight subjects.

2. Y1R, Y2R, and Y5R mRNA expressions are differently expressed between subcutaneous and visceral adipose tissues.

3. There were significant positive or negative correlations between expressions of Y1R, Y2R, and Y5R with clinical data and peripheral metabolic factors.

Objectives

1. Primary Objectives

1.1 To compare Y1R, Y2R, and Y5R mRNA expressions between normal weight and obese humans in subcutaneous and visceral fat tissues

1.2 To compare Y1R, Y2R, and Y5R mRNA expressions between subcutaneous and visceral adipose tissues in subjects

2. Secondary Objectives

2.1 To determine levels of blood insulin, glucose in normal weight and obese human subjects

2.2 To determine correlations between Y1R, Y2R, and Y5R mRNA expressions with SBP, DBP, body weight, BMI, blood leptin, glucose, insulin, and HOMA-IR

This study demonstrated the expressions of Y1R, Y2R, and Y5R mRNA and comparisons were made between obese and normal weight subjects as well as between subcutaneous and visceral adipose tissues. Moreover, blood glucose, insulin, and HOMA-IR were determined. Correlations of the gene expressions with serum leptin, clinical parameters, and peripheral metabolic factors were also analyzed. The results are shown as follows:

1. BMI, SBP, DBP, plasma insulin levels, and HOMA-IR were significantly greater in obese than normal weight humans.

2. To compare between obese and normal weight subjects, Y5R gene expression in both subcutaneous and visceral adipose tissues was higher in obese group than in normal weight group. In visceral adipose tissue, Y1R mRNA expression was greater but Y2R was lower in obese than in normal weight group. However, in subcutaneous fat tissue, there was no statistically significant difference of Y1R and Y2R between obese and normal weight subjects.

3. Comparisons between subcutaneous and visceral adipose tissue showed that Y1R mRNA was lower in visceral fat when compared to subcutaneous fat in obese, normal weight, and overall subjects without significant difference for Y1R in normal weight subjects. Y2R mRNA was higher expressed in subcutaneous than visceral fat tissue in obese subjects. There was similar expression between subcutaneous and visceral fat for Y2R mRNA in normal weight and overall subjects as well as for Y5R mRNA in obese, normal weight, and overall subjects.

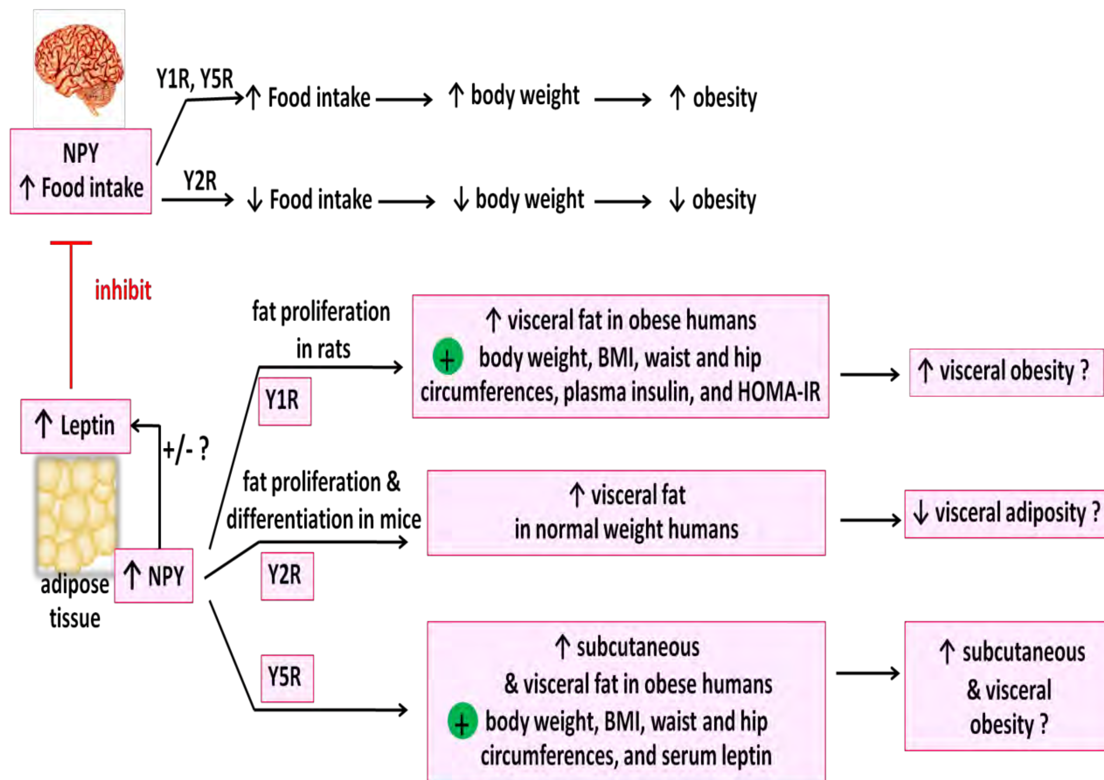
4. There were positive correlations of age with body weight and BMI. Body weight and BMI had positive correlations with insulin and HOMA-IR. SBP was positively correlated with body weight, BMI, insulin, glucose and HOMA-IR.

5. Y1R gene expression in visceral adipose tissue was positively correlated with body weight, BMI, waist circumference, hip circumference, insulin, and HOMA-IR.

6. A strong positive correlation was existed between Y2R mRNA expression in subcutaneous and visceral adipose tissues.

7. Y5R mRNA expression in visceral fat tissue was positively correlated with Y1R mRNA expression in visceral fat, body weight, BMI, hip circumference, and serum leptin levels. In subcutaneous fat, there was a positive correlation between Y5R and Y2R mRNA.

In conclusion, this study revealed that obese people have higher SBP, DBP, insulin levels, insulin resistance, and Y5R mRNA expression in both subcutaneous and visceral adipose tissue, and Y1R mRNA in visceral adipose tissue. Y2R mRNA in visceral fat tissue was higher expressed in normal weight subjects than obese subjects. Up-regulation of Y1R especially in visceral adipose tissue had positive correlations with body weight, BMI, waist and hip circumferences, insulin and HOMA-IR. Y5R mRNA expression in visceral adipose tissue was positively correlated to body weight, BMI, waist and hip circumference, and serum leptin levels. Thus, clinical and anthropometric data might be used as indicators of Y1R or Y5R mRNA in visceral adipose tissue which are closely associated with adiposity and risks of metabolic syndrome. Further investigation about blocking or activating specific receptor subtypes, i.e. Y1R, Y2R, and Y5R expressed in adipose tissue, may reveal new strategies for risk reduction in metabolic syndrome as well as prevention or treatment of obesity.



The schematic diagram showing conceptual framework of NPY action via Y1R, Y2R, and Y5R in the brain and adipose tissue. NPY plays a central role in appetite regulation and is inhibited by leptin (25, 46). Central NPY action via Y1R and Y5R is to increase food intake leading to increased body weight and obesity (38, 42, 43, 47, 48). NPY action via Y2R decreases food intake leading to reductions of body weight and obesity (45, 49). Peripheral NPY action includes increased fat proliferation via Y1R (16) and Y2R (15) in rodents and induced fat differentiation via Y2R in mice (15). NPY was also synthesized in adipose tissue and might stimulate (44) or inhibit (14) leptin secretion. In visceral adipose tissue, Y1R mRNA expression was higher in obese humans which might lead to increased visceral adiposity. Y2R mRNA expression was higher in normal weight subjects which might lead to reduced visceral adiposity. Y5R mRNA expression was higher in obese subjects in both subcutaneous and visceral fat which might lead to increased both subcutaneous and visceral adiposity. Therefore, NPY action on adipose tissue via Y1R and Y5R might be related to an increase in obesity while Y2R might be related to a decrease in visceral adiposity.

เนื้อหางานวิจัย

LITERATURE REVIEW

General introduction

Obesity, a state of increased body weight and more fat accumulation (1), causes many adverse health consequences at both individual and societal levels (1, 2). Body weight and energy storage in adipose tissue are decided by interaction of various factors including genetic, environmental, and psychosocial factor (1). Body mass index (BMI), calculated from the body weight in kilograms (kg) divided by height in meters squared (m^2), is a tool using for classification obesity levels (4). The classification of BMI in Asian adults was categorized in five groups which were less than 18.5 kg/m^2 as underweight, 18.5-22.9 kg/m^2 as normal weight, 23-24.9 kg/m^2 as overweight, 25- 29.9 kg/m^2 as obese grade I, and more than 30 kg/m^2 as obese grade II (4). For Caucasian populations, the BMI less than 18.5 was identified as underweight, 18.5-24.9 kg/m^2 as normal weight, 25.0-29.9 kg/m^2 as overweight, and more than 30 kg/m^2 as obese (2, 3). Growing obesity epidemic has become a public health problem throughout the world with 475 million adults and up to 50 million children being considered obese (data for 2010) (3). For Thai adults population, the prevalence of obesity with BMI more than or equal 25 kg/m^2 was nearly expanded 2 fold in the past two decades by rapid growth from 18.2% in 1991 to 28.1% in 2004 (5). It is well known that obesity predisposes to type II diabetes, cardiovascular diseases, hypertension, hyperlipidemia, degenerative arthritis, psychosocial impairment, and metabolic syndrome (2, 8, 11, 12) which is associated with abdominal obesity, high levels of blood sugar, lipid profiles, Homeostatic Model for Assessment of Insulin Resistance (HOMA-IR), systolic blood pressure (SBP), and diastolic blood pressure (DBP) (9, 10). The criteria for diagnosis of metabolic syndrome according to the Adult Treatment Panel III (ATPIII) includes the presence of defined abnormalities in any 3 of 5 measures which are waist circumference more than or equal 102 cm in men or 88 cm in women, triglyceride more than or equal 150 mg/dl, HDL-cholesterol less than 40 mg/dl in men or 50 mg/dl in women, blood pressure more than or equal 130/85 mmHg, and fasting plasma glucose more than or

equal 100 mg/dl (or diabetes) (50). For South Asians populations, cutoff points of waist circumference was accepted at more than or equal 90 cm for males and more than or equal 80 cm for females (51). Therefore, the adverse health consequences resulting from obesity need effective prevention and treatment. A better understanding of energy balance and regulation of food intake is required to unravel the complexity of obesity.

Major complex factor of obesity is control of energy metabolism (52) with excessive dietary intake and less energy expenditure causing fat accumulation and weight gain in humans (1). The energy expenditure consists of 70% of basal metabolic rate (BMR), 5-10% of physical activity, and other factors including adaptive thermogenesis and the energy cost of metabolizing and storing food (53). It has been revealed that appetite controls food intake (54) while feeding behavior is controlled by appetite, hormonal factors, psychological, visual, olfactory, and cognitive inputs, and also by neural signals via the vagus nerve (1, 26). Control of food intake is divided into short term regulation (meal-related determinants of food intake) and long term regulation (1, 55-57). The short term regulation includes gut distension transmitting signals through vagus nerve toward the nucleus tractus solitarius of brain stem resulting in satiety (1, 12, 58) as well as release of gut peptide hormones causing initiation or termination of food intake such as ghrelin, Cholecystokinin (CCK), (peptide YY) PYY_{3-36}, and glucagon like peptide 1 (GLP-1) (1, 59, 60). These signals are transmitted via vagus nerve or conveyed to brain stem or the hypothalamus (25, 55, 56, 61). The hormone ghrelin, secreted mainly from P/D1 cells lining the fundus of the stomach (62), increases progressively during fasting and declines to a nadir within an hour of refeeding (63). After eating, orexigenic pathway was inhibited by short term satiety signals including CCK, PYY_{3-36}, and GLP-1 (25, 56). Moreover, leptin secreted from stomach (64-66) was released in response to food intake (65, 66) or administration of satiety factors such as CCK (65), gastrin, and secretin (65, 67) suggesting a role of leptin in the short-term food regulation (68). Furthermore, postprandial insulin response was also related to short-term appetite regulation by increased satiety in humans (59, 69, 70). In long term regulation that reflects energy store or levels of adiposity, leptin and insulin act at the brain as peripheral satiety hormones that inhibit food intake (25, 46, 56). Leptin, secreted from adipocytes (71), reduces food intake, increases energy expenditure (72) and regulates neuroendocrine function (73). Insulin, secreted from pancreatic β cells,

rises in proportion to body adiposity (74, 75) and performs as a hormonal regulator of food intake and energy balance in rats (76-78), baboons (79), sheep (80), marmots (81), and humans (82). Thus, a linkage between central nervous system (CNS) and peripheral signals is an important factor of regulation of food intake and energy balance.

Appetite regulation

The appetite regulation is an orchestra of interaction between peripheral signals and the CNS especially in hypothalamus (25, 26). Many hypothalamic areas play a crucial role in feeding control including the arcuate nucleus of the hypothalamus (ARC), paraventricular nucleus (PVN), ventromedial nucleus of hypothalamus (VMH), lateral hypothalamus (LHA), and perifornical area (PFA) (26, 46, 83). Previously, the VMH was called satiety centre because its actions were shown in feeding inhibition and energy expenditure stimulation (84). On the other hand, the LHA was famous as feeding center or hunger center (26, 85) because lesions of the LHA caused hypophagia, hypermetabilic state, and weight loss (86-88). In the ARC, there are 2 neuronal populations including orexigenic neuronal populations containing neuropeptide Y (NPY)/ agouti-related peptide (AGRP) neurons, and anorexigenic neuronal populations containing pro-opiomelanocortin (POMC) and cocaine-and amphetamine-related transcript (CART) neurons (25, 26, 46, 56). The first-order neurons mediate and integrate the first contact of peripheral signals then transmit signals toward the second-order neurons resulting in signal modification (25, 26, 46, 56). Hypothalamus transmits signals to prefrontal cortex and other association cortices leading to food intake modulation (81). The LHA and perifornical area (PFA) areas contain orexins and melanin-concentrating (MCH) neurons which are orexigenic second order neurons (25, 26, 46, 56). In the PVN region, there are 2 anorexigenic second order neurons consisting of corticotrophin-releasing (CRH) neurons and thyrotropin releasing hormone (TRH) neurons (25, 26, 46, 56). Previous studies in mice reported that icv injection of ghrelin increased expression of hypothalamic NPY mRNA (89) and circulating ghrelin also increased expression of NPY/agouti-related peptide (AGRP) in the hypothalamus (90, 91) suggesting an important role of ghrelin in food intake stimulation. The peripheral

signals, leptin and insulin, act via their receptors on 2 different classes of neurons in the ARC of the hypothalamus by inhibiting the first-order orexigenic neuron populations and stimulating the first-order anorexigenic neuron populations (25, 26, 46, 56) which then send integrating satiety signals toward the second-order neuron. Thus, leptin decreases expression of orexigenic neurons, but increases expression of anorexigenic neurons resulting in decreased food intake (25, 26, 46, 56). Previous studies showed that administration of leptin reduced hypothalamic NPY mRNA expression in mice (92, 93), rats (94, 95), sheep (73), and humans (96). The interaction of peripheral signals, leptin and insulin, and hypothalamic neurons regarding to appetite regulation. The peptides involving in appetite regulation are divided in 2 different groups which are orexigenic and anorexigenic peptides. Generally, total body fat content is preserved in a constant level (97) explained by a "lipostatic" model defined as peripheral signals generated in proportion to body fatness that act on hypothalamus to regulate food intake and energy expenditure (98). Increased obesity with excessive fat mass leads to increased leptin (and insulin) levels and then decreased food intake resulting in weight loss and restoration of fatness and *vice versa* (98).

Neuropeptide Y

Neuropeptide Y (NPY), a 36 amino acid peptide (99), is a member of structurally related peptides family including peptide YY (PYY), and pancreatic polypeptide (PP) (100-103). PYY, and PP were appeared to be expressed in gut tissues (100, 101) while NPY was mainly expressed in the brain, especially in the hypothalamus (17, 21). NPY has first discovered in porcine brain by Tatemoto et al. since 1982 (99, 104) and was showed a potent stimulator of feeding in many species such as Siberian hamsters (105), rats (106, 107), and mice (108). This neuropeptide was named NPY because N- and C-terminus consisting of tyrosines (Y is an abbreviation of tyrosine) and amino acid sequences were found as follows;
Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2 (99). NPY amino acid sequences were presented homology with PYY (70%) and homology with PP (50%) (99).

NPY is abundantly expressed in the arcuate nucleus (ARC) of the hypothalamus (17-24) and in other areas of the central nervous system (CNS) including cerebral cortex, hippocampus, amygdale, nucleus of the solitary tract, locus coeruleus, nucleus acumbens, and limbic system (17-23, 109-114). NPY co-localization with catecholaminergic neurons were observed in rat (115, 116) and guinea pig (117) brain stem and human hypothalamus (118). NPY signals were transmitted from the ARC to other hypothalamic areas including PVN, VMH, dorsomedial hypothalamus (DMH), suprachiasmatic nucleus, median eminence (ME), anterior hypothalamus, and posterior hypothalamus in rats (110), hamters (112), and monkeys (119). NPY induces weight gain (120-124) and adiposity (120-122) by increasing appetite and vagal activity as well as suppressing thermogenic mechanism and sympathetic outflow (48, 125-127). After intracerebroventricular (icv) administration of NPY, food intake was stimulated in monkeys (128), broiler, and layer chicks (129), and rats (130). Chronic administration of NPY stimulated hyperphagia, increased basal insulin levels (131), increased body fat, and increased rate of body weight gain (120) in rats. Previous studies showed that NPY mRNA in ARC of the hypothalamus was highly expressed in lean sheep when compared with normal weight sheep (132). In addition, fasting significantly increased hypothalamic NPY mRNA in mice (133), rats (134, 135), hamsters (136), Brazilian flounder (137), and sheep (138). NPY mRNA expression in the hypothalamus was statistically significant increased in dietary induced obesity (DIO) rats when compared with dietary induced obesity-resistance (DIO-R) and chow food (CF) rats (139) in accord with another publication showing higher hypothalamic NPY mRNA expression in DIO mice than diet-resistant (DR) and low-fat-fed (LF) mice (140). Hypothalamic NPY mRNA expression was reduced in developmentally overfed adult mice (141) as well as long term consumption of the high fat diet in mice showed reduction of hypothalamic NPY concentrations (142). In addition, NPY levels were detected in human cerebrospinal fluid (CSF) and plasma (143, 144) without statistical difference between obese and normal weight subjects in both CSF and plasma (143). NPY levels were 3 fold higher in CSF when compared with the blood stream and there was no correlation between CSF and plasma NPY levels (143) while another report presented that NPY levels in CSF was only 2 fold higher than in plasma (144).

NPY is also synthesized and released peripherally in adrenal gland, adrenal chromaffin cells (27), sympathetic ganglia, tissues receiving dense

sympathetic innervations (28-32), and endothelial cells (33, 34). It has been revealed that NPY released from sympathetic junctions was associated with sympathetic activity e.g. vasoconstriction (102, 145) and it appeared to potentiate isoproterenol (β-adrenergic receptor agonist) action (146). Other peripheral functions of NPY include regulation of mood regulation, angiogenesis, and fertility (145). Previous study showed that stress increased NPY levels in platelet-rich plasma in rats (147) and NPY promoted angiogenic activity by increased migration, proliferation, and tube formation of human endothelial cells (33). Many studies regarding relation of NPY gene and obesity have been investigated becoming an interesting issue. There was an evidence that both NPY knockout male and female mice have less body weight and percent body fat than wild-type mice in high-fat diets stage (148). After fasting and then refeeding, body weight and food intake were reduced in NPY knockout mice when compared with controls (148) suggesting that lack of NPY prevented weight gain. Interestingly, recent studies showed NPY mRNA expression was detected in adipose tissues such as visceral adipose tissue of rats (16), subcutaneous adipose tissue of mice (15), and visceral and subcutaneous adipose tissues of humans (13, 14). NPY protein expression was detected in human adipocytes, abdominal subcutaneous, omental, and thigh adipose tissues with expression in abdominal subcutaneous adipose tissue being twofold higher than either paired omental or thigh adipose tissues (14). NPY mRNA expression in visceral adipose tissue was higher in leptin receptor-deficient obese Zucker rat than in lean rats (16) consistent with up-regulation of NPY mRNA in subcutaneous abdominal fat of obese leptin-deficient (ob/ob) mice when compared with controls (15). In addition, NPY mRNA in visceral fat tissue was increased by 6 fold in offspring of pregnant rats fed low-protein diet with addition of carbohydrate, the early-life programmed rat model of visceral adiposity, when compared with control rats. High fat and high sugar (HFS) diet combined with cool stress treatment also induced NPY mRNA expression in subcutaneous abdominal fat pads of mice (15). Furthermore, in immunohistochemical analysis, NPY antibody was found in the cytoplasm of human adipocytes (14). For NPY levels in the circulation, plasma NPY concentrations were not significantly different between obese and normal weight subjects (104). Treatment of anorexia nervosa girls with hypercaloric diet, psychotherapy, vitamins supplementation significantly decreased serum NPY levels, but increased body weight, BMI, and serum leptin levels at follow-up after

approximately 7 months of treatment when compared to baseline at the admission to the hospital (120). Previous experiment in rats showed that intracerebroventricular injection of NPY induced up-regulation of lipoprotein lipase (LPL) which is a key enzyme in lipogenesis suggesting antilipolytic potential of NPY (149). In addition, human adipocytes treated with recombinant (rh) NPY reduced glycerol release (14), an index of lipolysis, in accordant with NPY stimulated dose-dependent inhibition of lipolysis in human and dog adipocytes (150), confirming an antilipolytic action of NPY. NPY also stimulates fat proliferation via Y1R and Y2R (15, 16) and fat differentiation via Y2R (15) in rodents. As a result, roles of peripheral NPY on adipose tissue include increased fat differentiation, proliferation, and decreased lipolysis acting via its receptor.

Neuropeptide Y receptors

NPY is mediated via a family of G-protein coupled receptors known as Y receptors (35) consisting of at least seven subclasses of receptors which are Y1 receptor (Y1R) to Y7 receptor (Y7R) (36, 37), through intracellular Ca^{2+} and cyclic adenosine monophosphate (cAMP) (37, 151, 152). Y1R, Y2R, and Y5R are abundantly expressed in the brain of rats (38, 39), mice (35, 40), and humans (24, 41) and play a major role in feeding regulation (42). Y1R (41), Y2R (15), and Y5R (41) are also found in periphery such as adipose tissue. Neuropeptide Y3 receptor (Y3R) was illustrated in pharmacological studies in bovine and rat, but there was no report in human (24). Y4R greater affinity for PP than for NPY and PYY (24, 35) and was exhibited in the brain of rats (153, 154), and humans (155). Y6 receptor (Y6R) was expressed in the hypothalamus, liver, and kidney of chicken (36) while there was no clear physiological effect of Y6R (24). Y7R was only discovered in adrenal gland of chicken (36) and in the brain, eye, and intestine of zebrafish (156).

Neuropeptide Y1 receptor

Neuropeptide Y1 receptor (Y1R), highlighted in regulation of feeding, was expressed in several thalamic nuclei, hippocampus and in the hypothalamus of rats, mice (35) and humans (24). Moreover, Y1R mRNA was detected peripherally in

adrenal gland, kidney, heart (41, 157), uterus, skin, pancreas, thyroid gland (41), colon, and placenta (157). The potential functions of Y1R were demonstrated via stimulatory effect of NPY on hyperphagia, vasoconstriction (35), heart rate, anxiety (158), and bone homeostasis (159). Previous study exhibited that hypothalamic Y1R mRNA levels was significantly increased in DIO rats when compared to DR and chow food rats (139). It has been reported that a single dose icv administration of Y1R agonist increased plasma insulin levels at 15 minutes, 1 hour and 2 hours after injection in satiated Long-Evan rats with and without the presence of food (160). NPY and Y1R agonist reduced insulin induced translocation of glucose transporter 4 (GLUT4) from intracellular stores to cell surface in 3T3-L1 adipocytes and Y1R antagonist prevent the reduction indicating that NPY increased insulin resistance via Y1R (161). However, another study found that both Y1R deficient male and female mice increased body weight and plasma insulin levels at age 15 and 24 weeks (162). Administration of Y1 receptor antagonist in mice reduced body weight and food intake (42) consistent with the experiment in Sprague-Dawley rats showing that intravenous (iv) (3 and 10 mg/kg) and icv injection (30 and 100 μg) of selective Y1 antagonist, J-115814 reduced food intake induced by icv NPY administration at 5 μg (43). The effect of the selective Y1 antagonist, J-115814, at 10 and 30 mg/kg was demonstrated to be suppressed nocturnal food intake in leptin receptor-deficient (db/db) mice and C57BL/6 mice (43). It has been reported that NPY-induced food intake was significantly decreased in Y1R knockout mice but not in Y5R knockout mice when compare to wild type mice (163) suggesting dominant role of Y1R on NPY-induced feeding. Interestingly, recent studies showed that Y1R mRNA was also expressed in rat adipocytes (16), human subcutaneous adipocytes (41, 44), and subcutaneous abdominal adipose tissue detected by realtime-PCR or microarray analysis (41) and Y1R protein was found in human subcutaneous, visceral, and thigh adipose tissues (41) with highest expression of Y1R in subcutaneous adipose tissue being at least 5 fold higher than in other tissues (including brain, adrenal gland, and uterus) (41). Previous study showed that NPY stimulated proliferation of primary cultures of rat preadipocytes as well as murine 3T3-L1 preadipocytes cell via Y1R and the activation was through extracellular related kinase 1/2 (ERK1/2) (16). The strong antilipolytic effect of NPY was blocked by specific Y1R antagonists (44) enhancing the antilipolytic effect of NPY acting through Y1R. In addition, the effect of NPY induced leptin production in human cultured differentiated adipocytes was

blocked by selective Y1R antagonist (44) indicating that NPY modulation of leptin release is via Y1R. In conclusion, actions of Y1R in obesity include increased feeding, enhanced fat proliferation as well as antilipolysis.

Neuropeptide Y2 receptor

Neuropeptide Y2 receptor (Y2R) was localized in several brain areas in rats (39) and humans (24), especially in the hippocampus, hypothalamus, and brain stem (39). It is well known that Y2R has equally high affinity to C-terminus segment of NPY and PYY (164) with NPY_{1-36} and PYY_{3-36} being the major forms in circulation (61). Binding of the PYY_{3-36} to the hypothalamic Y2R was involved in the induction of satiety (35, 61). The study in rats and mice showed that peripheral injection of Y2R agonist (PYY_{3-36}) inhibited food intake and reduced weight gain (45, 49). Furthermore, it has been revealed that intraperitoneal injection of Y2R agonist (BT-48) showed dose-dependent inhibition of ad libitum food intake in C57BL/6 mice and also reduced food intake at 4 and 6 hours after injection in fasted mice (165). Peripheral injection of PYY_{3-36} reduced caloric intake at buffet lunch and decreased appetite evaluated by visual-analogue scores when compared to control in both obese and lean subjects (166). Specific deletion of Y2R at ARC of the hypothalamus increased food intake and body weight compared to controls (158). However, deletion of NPY-neurons specific Y2 receptor gene resulted in no change in food intake and body weight in both male and female mice (167). The study in rats showed that icv administration of NPY and PYY induced changing of duodenal motility from fed (postprandial) patterns into fasted (interdigestive) patterns through Y2R by increased irregular contraction and frequency waves (168). Previous study showed that hypothalamic Y2R mRNA was significantly decreased in diet-resistance (DR) mice when compared to DIO mice and low-fat-fed (LF) mice (140) consistent with reduced Y2R mRNA in the hypothalamus of DR rat when compared to rat fed with chow food group (139). In the periphery, Y2R is located on the terminals of sympathetic and parasympathetic neurons in rats (24), tongue epithelium, colon in mice (169), vessels, endothelial cells, preadipocytes of mice and humans and in subcutaneous abdominal fat pads in mice (15). The peripheral functions of Y2R were promoted in the field of proliferation and differentiation of mouse 3T3-L1 preadipocytes as well as stimulating angiogenesis of capillaries in adipose tissue in

human and mouse (15). Moreover, it has been reported that 50% reduction of adipose tissue weight and volume were presented in both obese and lean mice treated with Y2R antagonist (BIIE0246) (15). Preadipocyte differentiation of mice was stimulated by NPY mimicking the effect of insulin action by lipid filling of new adipocytes and this effect was blocked by Y2R antagonist (15). Y2R mRNA was remarkably up-regulated in subcutaneous abdominal fat of obese leptin-deficient (ob/ob) mice compared with controls (15) and long term combination of high fat diet and cold stress increased Y2R mRNA in subcutaneous abdominal fat tissues in mice (15). Chronic stress also induced NPY release and up-regulated Y2 expression in visceral fat in high-fat, high-sugar fed mice, resulting in metabolic syndrome-like symptoms of abdominal obesity, inflammation, hyperlipidemia, hyperinsulinemia, glucose intolerance, hepatic steatosis, and hypertension. Interestingly, fat accumulation and metabolic complications can be prevented or reversed by pharmacological Y2R inhibition at local intra-fat or adenoviral Y2R knock-down resulting apoptosis of endothelial and fat cells in mice (170). Moreover, previous study reported that sum weight of white adipose tissue was significantly decreased in Y2R knockout mice when compared to wild type mice, but the energy expenditure and physical activity did not show the different significance (171). Combined treatment of Y2R agonist and Y5R antagonist was significantly decreased fat mass whereas Y2R agonist alone tended to decreased fat mass by 12% in DIO mice (45). In conclusion, central effect of Y2R appeared to reduced food intake and body weight whereas peripheral function of Y2R involved in increased fat proliferation and differentiation. However, the effect of NPY induced lipid filling of new adipocytes was blocked by Y2R antagonist. So, the effect of Y2R on adipose tissue mass is still inconclusive.

Neuropeptide Y5 receptor

Neuropeptide Y5 receptor (Y5R) mRNA and protein were detected centrally in the brain including hypothalamus of rat (38), mouse (40), and human (41) and peripherally in many tissues such as adrenal gland, kidney, testis, pancreas, vascular smooth muscle cells, cardiomyocytes in rodent and human (24, 41), skeletal muscle, skin, ovary, thymus gland, heart, uterus, thyroid gland, and lung in humans (15). Interestingly, recent study by microarrays showed that Y5R mRNA was expressed in human in both large and small subcutaneous adipocytes and abdominal subcutaneous tissue (41). The central role of Y5R is implicated in the regulation of

food intake, energy homeostasis, memory processing, and emotional functions (35). Intracerebroventricular (icv) injection of Y5R-selective agonist significantly increased food intake (38, 47, 48), increased body weight (47, 48), reduced energy expenditure (48), and suppressed circulating levels of thyroid hormones (T3 and T4) when compared with controls (47) in rats and enhanced hyperphagia, body weight gain, increased adipose tissue weight, hypercholesterolemia, hyperinsulinemia, and hyperleptinemia in C57BL/6J mice (172). Treatment of Y5R antagonist orally decreased NPY-induced body weight and cumulative food intake in mice as well as chronic co-administration of PYY_{3-36} (subcutaneous injection) and Y5R antagonist (p.o.) reduced body weight and food intake in DIO mice (45). Moreover, previous study showed that hypothalamic Y5R mRNA was increased in DIO rat when compared to DR rat and rat fed with chow food (139). Interestingly, the study in mice showed synergistic interaction between the Y1R and Y5R in increasing food intake and body weight (42), but both Y1R and Y5R knockout mice displayed late onset obesity (173) with hyperinsulinemia and hyperphagia found in Y5R knockout mice (24, 163, 173). Late onset obesity in these mice is associated with up-regulation of hypothalamic NPY mRNA (174) and/or compensation of the Y5R or Y1R subtypes in the Y1R or Y5R knockout mice, respectively (42). Collectively, Y5R plays a major role on an increase in food intake and body weight, and a reduction of energy expenditure.

Adipose tissue

Adipose tissue is a loose connective tissue containing adipocytes and plays a key role in energy storage in the form of triglyceride (175). Fat tissue contains 50% adipocytes and other components including vascular elements, preadipocytes, fibroblasts, mast cells, macrophages, nervous elements, and mesenchymal cells (175). There are two common types of adipose tissue in mammals which are white adipose tissue (WAT) containing white adipocytes and brown adipose tissue (BAT) containing brown adipocytes (175, 176). WAT is mainly involved in energy storage and hormone secretion while BAT is implicated in thermogenic mechanism (176). Adipose tissue has been identified as an endocrine gland since discovery of leptin secretion in 1994 (177) and also as paracrine or autocrine gland with many receptor expressions (178-180). Numerous hormones were secreted from adipose organ

including leptin (177), tumor necrosis factor-alpha (TNF-α) (181), interleukin-6 (IL-6) (181, 182), adiponectin (183), resistin (184), plasminogen activator inhibitor-1 (PAI-1) (185), monocyte chemoattractant protein-1 (MCP-1) (186), angiotensinogen (AGT) (187), and angiotensin converting enzyme (ACE) (187).

Moreover, there are two different classifications of adipose tissue depots which are subcutaneous and visceral adipose tissue (180, 188, 189). In anatomical location, main areas of subcutaneous adipose tissue are located at anterior abdominal wall, femerogluteal region, and back (180) and approximately 80% of whole body fat is identified as subcutaneous fat (175, 189). Intra-abdominal fat compartment is identified as visceral or omental adipose tissue (180) and highly associated with metabolic risks (190, 191). For venous blood supply, free fatty acids (FFA) and adipokines from visceral fat was directly drained to the liver via the portal vein while venous drainage of subcutaneous fat was transported through systemic vein (180). Drainage of FFA directly to portal vein caused FFA stimulated very low density lipoproteins (VLDL) secretion by the liver (192) and increased hepatic gluconeogenesis (193). Mean subcutaneous adipocyte size was larger than mean omental adipocyte size (194, 195). However, another study revealed that visceral adipose tissue contains a lot of large adipocytes whereas subcutaneous adipose tissue contains small adipocytes which have more insulin sensitivity than large adipocytes (196, 197). Size of omental adipocyte was positively correlated with HOMA-IR which is the degree of insulin resistance whereas there was no correlation between subcutaneous adipocyte size and clinical parameters (194).

The molecular and functional differences between two adipose tissue depots (subcutaneous and visceral fat) have been explained by various gene expressions. The study in obese humans showed that **leptin** gene expression was significantly greater in subcutaneous than visceral fat tissue (195, 198) and there was a positive correlation between age and leptin gene expressions in visceral adipose tissue but not in subcutaneous tissue (195). BMI was directly proportionate with leptin mRNA expression in subcutaneous fat (199) and there was a strong positive correlation between leptin levels and total body fat (200). Moreover, there was an inverse correlation between leptin and adiponectin gene expression in both subcutaneous and omental fat tissue while a positive correlation was found between leptin and TNF-α gene expression in subcutaneous fat tissue (198). However, other adipokines were higher expressed, synthesized and released from visceral adipose

tissue when compared to subcutaneous fat including **TNF-α** (181), **IL-6** (181, 182), **adiponectin** (183) and **angiotensinogen** (201). **TNF-α** was involved in increased adiposity, insulin resistance, and hypertension (202). In African-American subjects, TNF-α and **IL-6** protein secretions from adipose tissue were highly expressed in obese subjects when compared with lean subjects (BMI < 25 kg/m^2) (203). In Italian-Caucasian subjects, fasting plasma IL-6 levels were shown inversely proportionate with the rate of insulin-stimulated glucose disposal indicating that IL-6 might be reduced insulin sensitivity (204). Previous study showed that IL-6 and TNF-α reduced insulin sensitivity through long term inhibitory effects on the gene transcriptions of insulin-receptor substrate-1 (IRS-1), glucose transporter 4 (GLUT-4), and peroxisome proliferator-activated receptor gamma (PPAR-γ) (205, 206). **Adiponectin** mRNA expression in subcutaneous adipose tissue was decreased in type II diabetic patients and plasma adiponectin levels were declined in overweight, obese, and diabetic patients (207). The study in db/db mice showed that hepatic insulin-receptor substrate-2 (IRS-2) mRNA was increased in adiponectin treatment through a macrophage-derived IL-6-dependent pathway (208). **Angiotensinogen** was mainly expressed in the liver (209) and also expressed in adipose tissue (201) and previous study showed that SBP was decreased in HF-fed adipocyte angiotensinogen-deficient mice when compared with controls (210).

In adipose tissue, many receptors expression were found including **glucocorticoid receptors** (211), **androgen receptors** (212), **estrogen receptors** (212), **thyroid hormone receptors** (213), and **adrenergic receptors** (214). **Glucocorticoid receptor** was highly expressed in visceral fat depot (215) and involved in metabolic regulation (189, 215) and abdominal obesity (216). **Androgen and estrogen receptors** were greater density in visceral fat tissue than subcutaneous fat tissue (212). Estrogen induced up-regulation of α_2 adrenergic receptor subtype in human adipocytes via estrogen receptor-α (ER-α) leading to antilipolysis in subcutaneous fat depot but not in visceral fat depot (217). Previous study showed that androgen receptor gene was higher in intra-abdominal fat than subcutaneous fat in boys and subcutaneous fat distribution was lower in boys when compared to girls (218). It has been revealed that both **thyroid-stimulating hormone receptor (TSHR)** and thyroid hormone receptor-α1 subunit (TR-α1) gene expressions were higher in subcutaneous than visceral adipose tissues in obese subjects and protein expression of TSHR was lower in obese subjects than control subjects in both subcutaneous and

visceral adipose tissue (213). In hypothyroidism (Tshr$^{hyt/hyt}$) mice, insertion of TSHR gene increased uncoupling protein-1 (UCP-1) mRNA and protein expression in brown adipose tissue (BAT) revealing thermogenesis function of TSHR (219). Previous study showed that human abdominal visceral adipocytes were more sensitive to catecholamines stimulation via **adrenergic receptors** than subcutaneous adipose adipocytes leading to higher lipolytic rate (180, 220). Furthermore, β_3-selective adrenergic agonist (CL-316243) increased serum free fatty acid in treated mice when compared to control (221). Increased sensitivity of β_3-adrenoreceptor to catecholamine stimulation showed an increased of free fatty delivery into the portal venous system leading to hepatic gluconeogenesis and hyperinsulinemia (222). Visceral adipocytes were highly elevated in lipolytic activity (223) causing both an elevation of liver uptake free fatty acids (224) and gluconeogenesis (180, 225). This leads to a reduction of hepatic insulin extraction by inhibiting insulin breakdown (226) resulting in peripheral hyperinsulinemia (227-229). Furthermore, the influx of free fatty acids into the liver induces a decrease in skeletal muscle sensitivity to insulin leading to decreased glucose disposal and increased glucose-stimulated insulin secretion in normal subjects (189).

Collectively, NPY and NPY receptors which are Y1R, Y2R, and Y5R genes play a major role on regulation food intake and obesity. Each NPY receptor plays a different role in modulation of adiposity and fat accumulation. The differences of visceral adipose tissue and subcutaneous adipose tissue were illustrated in anatomy, gene expression, and function differences. Moreover, there was highly associated of visceral adipose tissue with lipolysis, insulin sensitivity, and gluconeogenesis. Various gene expressions were higher in visceral adipose tissue than subcutaneous adipose tissue and showed different expression between obese and normal weight subjects. In this study, we hypothesized that NPY and NPY receptors might be expressed differently between obese and normal weight subjects as well as between visceral and subcutaneous adipose. The results from this study will further elucidate different receptor subtype expressions in subjects with different BMI classification and correlations between the gene expressions with clinical data and peripheral metabolic factors. Manipulation of NPY and NPY receptors differently expressed in human adipose tissue in obese and normal subjects such as blocking or activating specific receptor subtypes may propose new strategies for obesity treatment.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University (si533/2009). All female subjects gave informed consent prior to the study. The sample size was determined using test of significant correlation between 2 factors calculated by setting $\alpha = 0.05$, $Z_{\alpha} = 1.96$, $\beta = 0.2$, $Z_{\beta} = 0.84$, H0: r = 0, and H1: r = 0.5. So, total number of subjects in this study was 30. The sample size was calculated as the following formula;

$$N = \left(\frac{(Z_{\alpha/2} + Z_{\beta})}{C} \right)^2 + 3$$

$$C = 0.5\ ln\ [(1+r)/\ (1-r)]$$

All 30 female patients who underwent abdominal surgery were recruited and divided into 2 groups, which were obese (n=17) (BMI > 25 kg/m^2) and normal weight (n=9) (BMI 18.5 – 22.9 kg/m^2). The other 4 subjects were overweight or lean and were recruited for correlation analysis. Subjects on endocrine therapy (e.g. steroids, hormone replacement therapy, thyroxine), and those who were pregnant, lactating, menopause, undergoing traumatic operations, had malignant diseases, or who underwent operations related to endocrine diseases and severe abdominal inflammation were excluded. In this study, male subjects could not be recruited because most of male patients who underwent open abdominal surgery were cancer or emergency operations which were fallen in the exclusion criteria. Other studies collecting visceral adipose tissue from open abdominal surgery were also done in female (14, 41). Phase of menstrual cycle of female subjects recruited in this study couldn't be controlled because most of the subjects had myoma uteri presenting with irregular menstruation.

Blood and tissue collection

Blood was collected during the fasting state before operation. Blood sample was divided into 3 tubes including 2 ml in sodium fluoride coated tube for glucose analysis, 2 ml in lithium heparin coated tube for insulin analysis and 6 ml in clot activator tube for NPY and leptin analysis. Both glucose and insulin analyses, blood samples were sent to the central laboratory of Department of Clinical Pathology Faculty Medicine Siriraj Hospital, Mahidol University. For NPY and leptin analyses, blood samples were incubated for 1 hour, balanced weight and separated phase by centrifugation at 5,000 rpm, 4 ◦C for 15 min. Then, serum samples were aliquoted and stored at -70 ◦C until analysis. Four to five pieces of 0.5 cm of each type of adipose tissues, which were abdominal subcutaneous (S) and omental (visceral-V) adipose tissues were collected during operation. For each subject, both subcutaneous and visceral adipose tissues were collected; as a result, there were 4 subgroups in this study as presented in Table 1.

Adipose tissues were immediately snap-frozen in liquid nitrogen to preserve RNA from RNase action and stored at -70 ˚C until gene expression analysis. A piece of 1 x 1 cm^2 of each type of adipose tissues, which were abdominal subcutaneous (S) and omental (V) adipose tissues was fixed in 4% paraformadehyde overnight, snap-frozen with liquid nitrogen and then kept at -70 ˚C until NPY protein analysis.

Table 1 Identification of groups

Group	Obese		Normal weight	
BMI	> 25 kg/m^2		18.5 – 22.9 kg/m^2	
Number of subjects	17		9	
Type of tissue	S	V	S	V

Methods

Demographic details and anthropometric measurements

Age, body weight, BMI, waist and hip circumferences, and the cause of operations were collected from subjects. According to the WHO guideline, waist

circumference was applied by measurement at level of the umbilicus with silent breathing in standing position and hip circumference was also measured in standing position at inter-trochanteric girth (230).

RNA extraction

Each adipose tissue sample (approximately 200-300 mg) was homogenized for RNA extraction by using 1 ml TRIzol® Reagent (Invitrogen™, Carlsbad, California, USA), according to the instructions of the manufacturer. The homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Then, 200 µl of chloroform was added and the tube was capped securely and shaken vigorously by hand for 15 seconds. The tube was incubated for 10 minutes at room temperature. Then, the sample was centrifuged (Labnet PrismTMR, Labnet international, Inc) at 12,000 × *g* for 15 minutes at 4°C. The sample was separated into a lower red phenol-chloroform phase-protein layer, an interphase-DNA layer, and a colorless upper aqueous phase. RNA remains in the aqueous phase. Then, the aqueous phase of the sample was removed by angling the tube at 45° and pipetting the solution out. The aqueous phase was placed into a new tube and 0.5 ml of 100% isopropanol was added to the aqueous phase. The tube was incubated at room temperature for 10 minutes and centrifuged (Labnet PrismTMR, Labnet international, Inc) at 12,000 × *g* for 1 hour at 4°C. The supernatant was removed from the tube, leaving only the RNA pellet. The pellet was washed, with 1 ml of 75% ethanol. The sample was mixed, and then centrifuged at 7500 × *g* for 5 minutes at 4°C. The wash was discarded and the RNA pellet was air dried for 10 minutes. The RNA pellet was resuspended in diethylpyrocarbonate (DEPC) water in total volume 20 µl. The extraction yield was quantified spectrophotometrically at wavelength of 260 nm (A260) and 280 nm (A280) by using NanoPhotometer™ (Implen, Ontario, New York, USA). A260 is used to determine RNA concentration whereas A280 determine protein concentration. The A260/A280 ratio used to assess RNA purity of our samples was more than 1.8 showing little protein contamination as a ratio of approximately 2.0 is generally accepted as "pure" for RNA (231). RNA was stored in -70°C freezer. The quality of RNA samples were assessed by electrophoresis through 2% denaturing agarose gel. For each well, 3 µl of RNA sample and 1 µl of loading dye (Thermo Scientific, Waltham, Massachusetts,

USA) were loaded. The gel was placed on electrophoresis chamber (Bio-Rad, Hercules, CA, USA) containing 1X Tris-acetate-EDTA (TAE) buffer and this chamber was connected to power supply (Bio-Rad, Hercules, CA, USA) at 100 voltage of electrical potential for 30 minutes. The gel was stained with ethidium bromide for 10 minutes and then washed with distill water for 15 minutes to visualize the 18S and 28S RNA bands under UV illumination using UV transilluminator imaging machine (Bio-Rad, Hercules, CA, USA) as shown in figure 1.

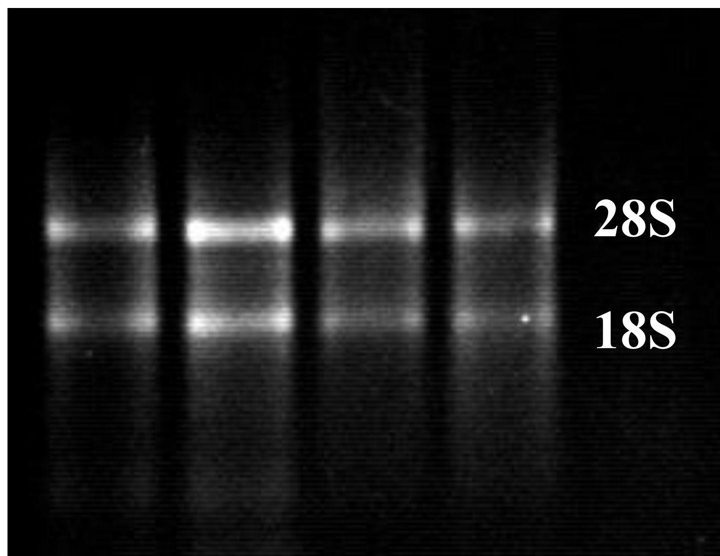


Figure 1 Gel electrophoresis revealing 28S and 18S bands of rRNA

Complementary DNA (cDNA) synthesis

About 1 µg of RNA was reverse transcribed to complementary DNA (cDNA) by using iScript™ cDNA Synthesis Kit (Bio-RAD, Hercules, California, USA) which is a modified MMLV-derived reverse transcriptase preblended with RNase inhibitor. In the iScript reaction mix, there is a blended of oligo(dT) and random hexamer primers. It is well known that eukaryotic mRNA contains poly(A) tail, so using oligo(dT) increase specificity binding between primer and mRNA. Random hexamer primers are useful for either long mRNAs, or transcripts with significant secondary structure. The components of one reaction in a total volume 20 µl are shown in Table 2. After mix thoroughly of all reagents, the cDNA was amplified as the following conditions: 5 minutes at 25 ºC, 30 minutes at 42 ºC, and 5 minutes at 85 ºC by using mastercycler gradiet (Eppendorf, Hamburg, Germany).

Table 2 iScript™ cDNA Synthesis protocol

Components	Volume per reaction (µl)
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Nuclease-free water	X µl
RNA template	X µl

Total volume 20 µl

Synthesis of Oligonucleotide primers

The oligonucleotide primers for real-time PCR were designed by the authors using published nucleotide sequences from PubMed database. All primers were designed with the exon to exon sequences to confirm their specificity to mRNA and were blasted to all species sequences to prove their specificity to genes of interest. The real-time PCR primer sequences are shown in Table 3.

Table 3 Oligonucleotide primers

Primers	Nucleotide sequences	Product size (base pairs)
Y1R	Forward-5'ATCATGCTGCTCTCCATTGTGGT 3'	222
	Reverse-5' GTTGAAGAAGAACTGCAAGTCTCTCT 3'	
Y2R	Forward -5'GGCCTACTGCTCCATCATCTTG 3'	228
	Reverse -5'CCCTGGGCATAGGGCACC 3'	
Y5R	Forward -5'CTGATAGCTACTGTCTGGACACT 3'	302
	Reverse-5'AGAGTTAAGTTGATCATCTCATTTTCTTC 3'	
LRP-10	Forward- 5' GATGGAGGCTGAGATTGTGCA 3'	169
	Reverse- 5' TGGAGTCATATCCTGGCGTAAG 3'	

Real-time Polymerase Chain Reaction (Real-time PCR)

Real-time Polymerase Chain Reaction (Real-time PCR) was performed to quantify mRNA expressions of NPY, Y1R, Y2R, Y5R, and low density lipoprotein

receptor-related protein 10 (LRP-10) and the primer sequences used in this study are shown in Table 3. LRP-10 was used as a reference gene because it is the most stably expressed gene in human adipose tissue (232). Real-time PCR was carried out using the reagents and protocol contained in the VeriQuest™ SYBR® Green qPCR Master Mix with ROX™ (Affymetrix, Santa Clara, California, USA) and the reaction mix is shown in Table 4. The 2X master mix contains chemically-modified VeriQuest Taq DNA Polymerase, $MgCl_2$, ultrapure nucleotides with an optimized dUTP:dTTP ratio, Uracil-DNA Glycosylase (UDG), SYBR Green, and ROX™ passive reference dye. Since the mix contains UDG, so carryover contamination from previous PCR experiment can be prevented by cleaving uracil base from uracil-containing DNA. Then UDG was inactivated by heat denaturation prior to actual PCR experiment. ROX™ passive reference dye is used to normalize fluorescent signal intensities. SYBR Green is fluorescent dye which binds to the minor groove of double strand DNA and increasing of fluorescence was detected all through the cycle. Each reaction was done in duplicate. After thorough blend of master mix, primers and DNA samples, all samples were spun down to collect contents without bubbles. The PCR amplification was performed by Agilent Mx3005P QPCR Systems (Agilent Technologies, Santa Clara, California, USA) under the following conditions: UDG treatment at 50 ºC for 2 min, Taq DNA polymerase activation at 95 ºC for 10 min, 40 cycles of DNA denaturing at 95 ºC for 15 sec, annealing at 57 ºC for 60 sec, and extension at 72 ºC for 30 sec. Amplification products of all genes were presented as amplification plots and the cycle threshold (C_T) of amplification products which are shown at the exponential phase of reactions as the following Figure 2-4. No template control (NTC) which are the PCR reaction without DNA template, was performed as negative controls and DNase, RNase-free water was used to replace DNA template to maintain the final volume of the reaction. cDNA converted from RNA extracted from human brain was used as a positive control. The actual RT-PCR products size was proved using DNA ladder (BioLabs Inc, Ipswich, Massachusetts, England) by electrophoresis through 2% denaturing agarose gels at 75 voltages for 100 minutes. For each well, 10 µl of DNA sample and 2 µl of loading dye (Thermo Scientific, Waltham, Massachusetts, USA) were loaded. The gel was stained with ethidium bromide for 30 minutes and then washed with distill water for 2 hours to visualize RT-PCR products size under UV illumination using UV transilluminator imaging machine (Bio-Rad, Hercules, CA, USA) as shown in figure 5.

Table 4 VeriQuest^{TM} SYBR$^{®}$ qPCR Master Mix protocol

Components	Volume (µl)
VeriQuest SYBR Green qPCR Master Mix (2X)	10.0 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
Template DNA	2.0 µl
Distilled water RNase, DNase free	7.0 µl
	total volume 20 µl

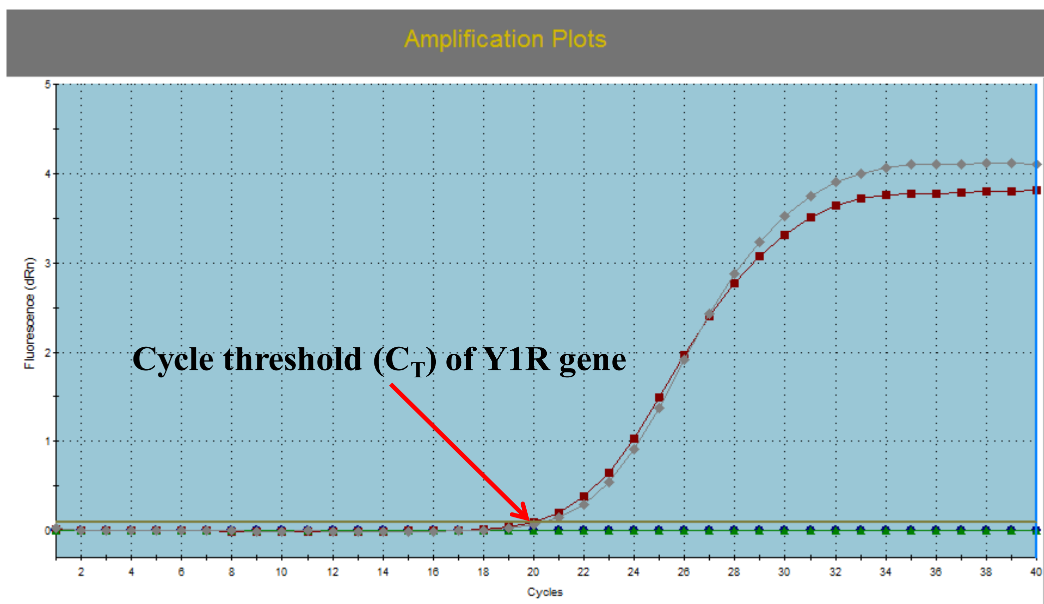


Figure 2 The amplification plots of Y1R gene. X axis is cycle number. Y axis is fluorescence signal; dRn is baseline-corrected normalized fluorescence on the reference dye.

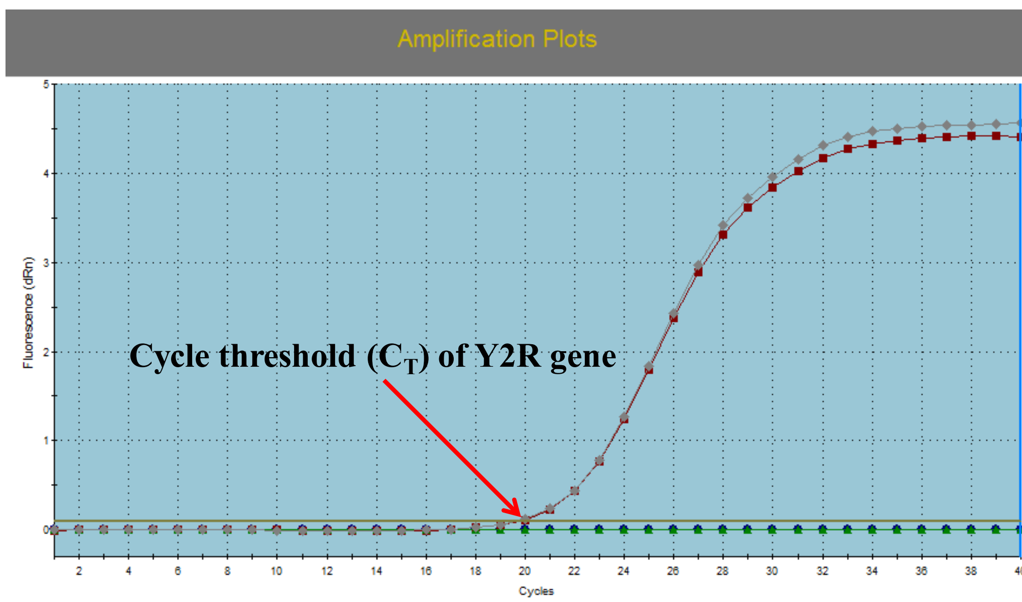


Figure 3 The amplification plots of Y2R gene. X axis is cycle number. Y axis is fluorescence signal; dRn is baseline-corrected normalized fluorescence on the reference dye.

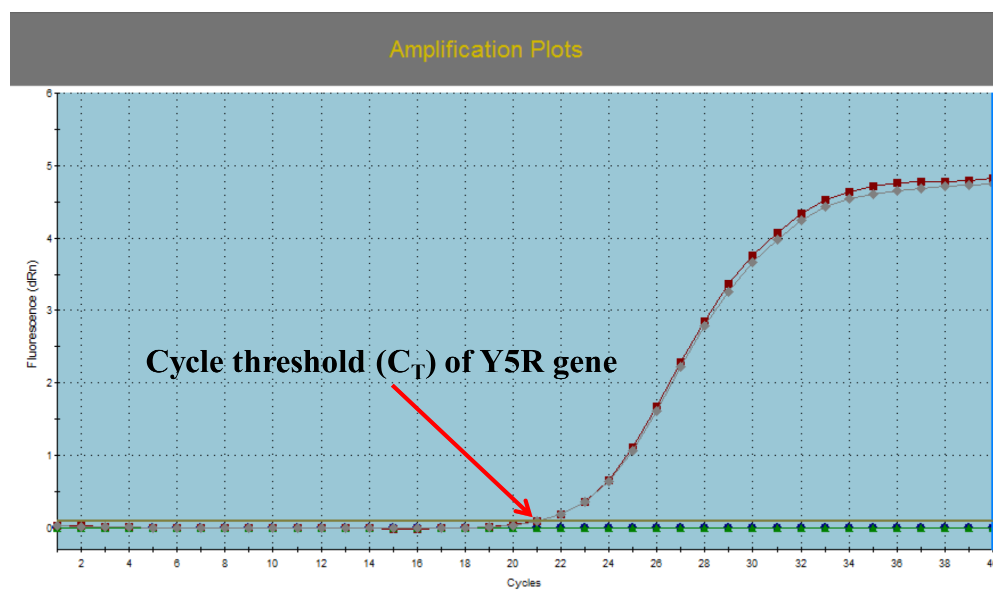


Figure 4 The amplification plots of Y5R gene. X axis is cycle number. Y axis is fluorescence signal; dRn is baseline-corrected normalized fluorescence on the reference dye.

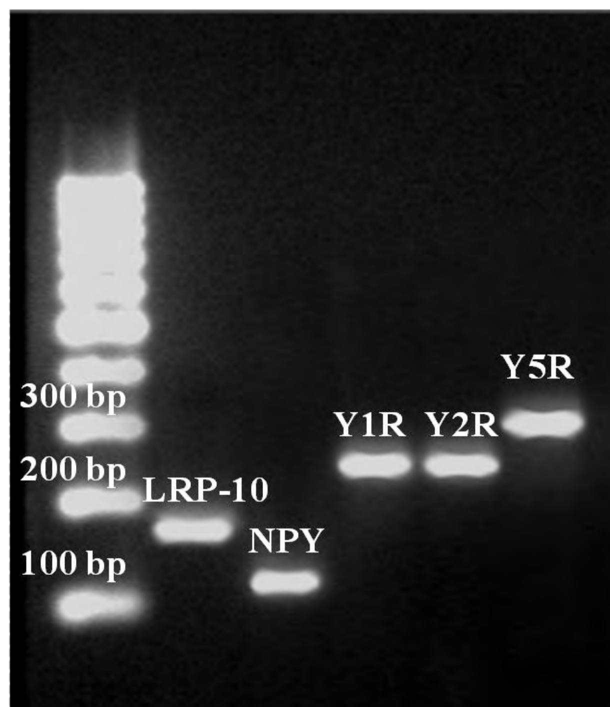


Figure 5 Gel electrophoresis revealing expression of LRP-10 (amplicon size 169 bp), NPY (amplicon size 102 bp), Y1R (amplicon size 222 bp), Y2R (amplicon size 228 bp), and Y5R (amplicon size 302 bp)

Efficiency of primer

To test efficiency of each primer, the DNA sample was diluted into 5 dilutions which were 2, 1:1, 1:2, 1:4, and 1:8 concentrations. The amplicons were amplified according to the VeriQuest^{TM} SYBR$^{®}$ Green qPCR Master Mix (Affymetrix, Santa Clara, California, USA) protocol and followed in conditions as previously described in topic 3.3.4 Real-time Polymerase Chain Reaction (Real-time PCR). The results of the real-time PCR reaction for Y5R shown as amplification plots are displayed in Figure 6. The standard curve with the C_T plotted against the log of the starting quantity of template of each dilution is shown in Figure 7. The R^2 value of linear standard curve should be more than 0.980 and the R^2 of our study was 0.9969. The slope was calculated from regression line and the efficiency (E) was calculated according to the equation; $E = 10^{-1/slope}$. Amplification efficiency is frequently presented as a percentage (% efficiency, %E), that is the percentage of template that was amplified in each cycle calculated by the following equation; %E = (E-1) x 100%. Efficiency close to 100% is the best indicator of robust reproducible assay and

the amplification efficiency of 90-105% is accepted. For example, for Y5R, the slope was -3.5354; then E equaled 1.9180213. As a result, %E was 91.80213 and was accepted as a robust, reproducible assay as shown in the calculations below.

$$E = 10^{-1/slope} = 10^{-1/3.5354}$$

$$E = 1.9180213$$

$$\%E = (E-1) \times 100\%$$

$$= (1.9180213-1) \times 100\%$$

$$= 91.80213$$

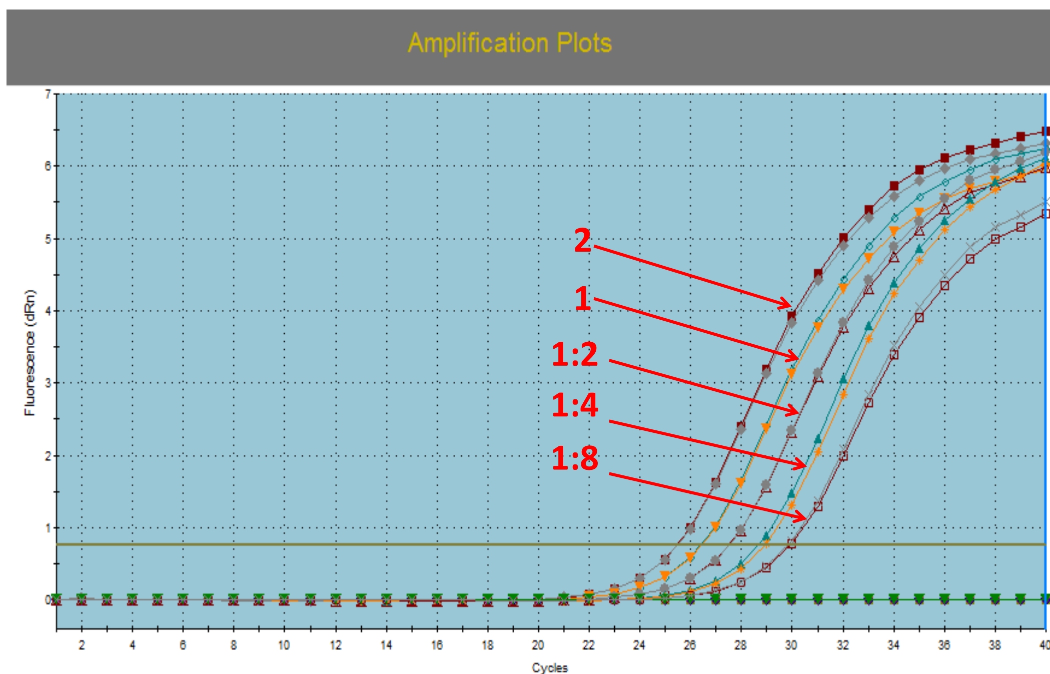


Figure 6 The amplification plots of Y5R gene showing amplification products of 5 dilutions of DNA from human adipose tissue. X axis represents cycle number. Y axis represents fluorescence signal.

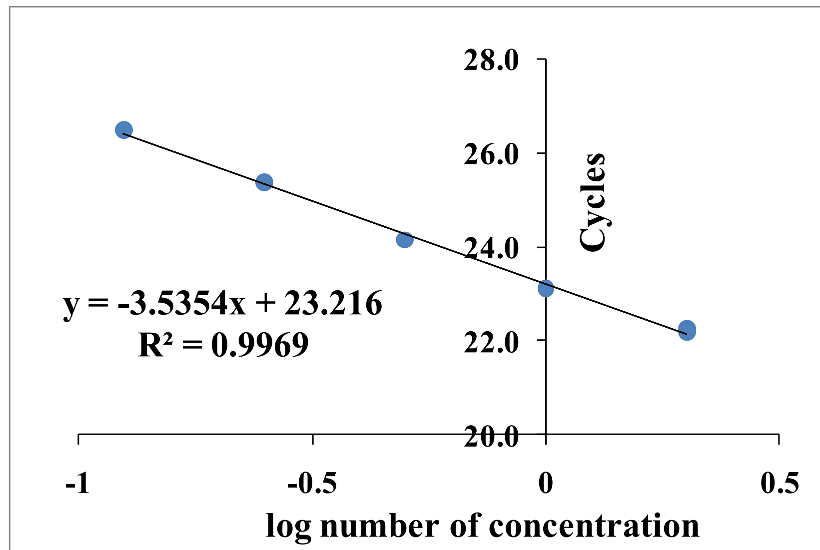


Figure 7 The standard curve of Y5R with the C_T plotted against the log of the starting quantity of template of each dilution

Real-time Polymerase Chain Reaction (Real-time PCR) analysis

The amount of DNA is theoretically increased in double in each PCR cycle, then after n cycle when n is a cycle number; the amplification product equals 2^n. $2^{-\Delta CT}$ method was applied as a comparative method of quantification due to the efficiency of each primer near 100%. ΔC_T is the difference between C_T of gene of interest and C_T of reference gene in the corresponding samples, so $2^{\Delta CT}$ was represented by the following procedure;

$$2^{\Delta CT} = 2^{\text{CT of gene of interest} - \text{CT of reference gene}}$$

An increase in ΔC_T represents a decrease in gene expression normalized with reference gene; therefore, adding negative to ΔC_T should be inversed determination. Thus, the equation was shown in $2^{-\Delta CT}$. Consequently, NPY, Y1R, Y2R, and Y5R gene expressions were calculated from the following formula;

NPY gene expression; $2^{-\Delta CT} = 2^{-(\text{CT of NPY gene} - \text{CT of LRP-10 gene})}$

Y1R gene expression; $2^{-\Delta CT} = 2^{-(\text{CT of Y1R gene} - \text{CT of LRP-10 gene})}$

Y2R gene expression; $2^{-\Delta CT} = 2^{-(\text{CT of Y2R gene} - \text{CT of LRP-10 gene})}$

Y5R gene expression; $2^{-\Delta CT} = 2^{-(\text{CT of Y5R gene} - \text{CT of LRP-10 gene})}$

Hormonal assay

Analysis of plasma glucose and insulin

Both plasma glucose and insulin were analyzed by the central laboratory of Department of Clinical Pathology Faculty Medicine Siriraj Hospital, Mahidol University by immunoturbidimetric assay (Hitachi, Chiyoda, Tokyo, Japan) for plasma glucose and by electrochemiluminescent immunoassay (Lincoln, Madera, California, USA) for plasma insulin. Fasting plasma glucose and insulin levels can be applied to determine levels of insulin resistance using the method of the HOMA-IR which is calculated by multiplication of fasting glucose (mg/dl) and fasting insulin (µU/ml) divided by 405. The cutoff of HOMA-IR levels for increasing metabolic syndrome shown in non-diabetic populations in Southeast Asia was 2.34 (233), so the HOMA-IR more than 2.34 was considered as insulin resistance in this study.

Analysis of serum leptin

Serum leptin levels were measured by commercial enzyme-linked immunosorbent assay (ELISA) Kit (Phoenix Pharmaceuticals Inc.) according to the manufacturer's guidelines. The range of leptin detection was 0.31-20 ng/ml and the minimum detectable concentration was 0.312 ng/ml. The immunoplate is pre-coated with anti-human leptin capture antibody with blockage of the non-specific binding sites. The human leptin in the sample or in the standard solution can bind to the capture antibody which is immobilized in the wells. After washing procedure, the biotinylated anti-human leptin primary antibody is added and the enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the yellow color is directly proportionate to the amount of human leptin in the standard solutions or samples. The principle of the ELISA assay is shown in Figure 8

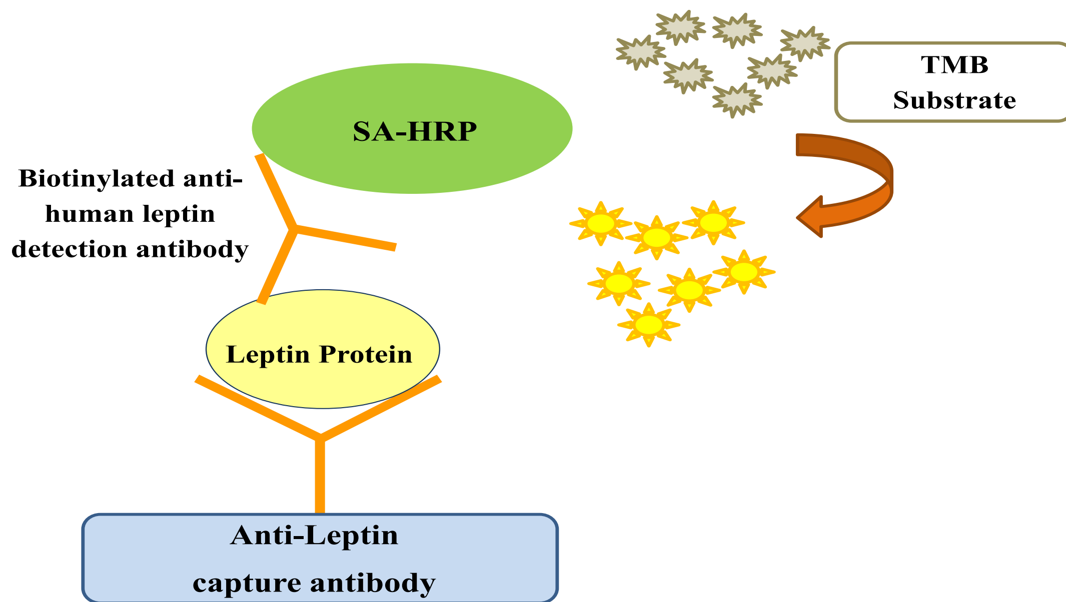


Figure 8 The principle of ELISA assay
(Adapted from PathScan® ELISA; http://www.cellsignal.com/ddt/elisa_line.html)

The kit was brought to room temperature and allowed to return to room temperature for 45 minutes. Firstly, 50 ml of 20x assay buffer was diluted into 1x assay buffer solution with 950 ml of distilled water and all serum samples were then diluted with 1x assay buffer with 1:5 dilution. Then, recombinant leptin standard was rehydrated with 1ml of 1x assay buffer, was incubated at room temperature for 10 minutes, thoroughly mixed with vortex, and then spun down. Leptin standard solutions were prepared as shown in Table 4.

Table 4 Preparation of peptide standard solutions of leptin

Standard number	Standard volume	1X Assay buffer	Concentrations
Stock	powder	1000µl	100 ng/ml
#1	200 µl of stock	800µl	20 ng/ml
#2	500 µl of #1	500µl	10 ng/ml
#3	500 µl of #2	500µl	5 ng/ml
#4	500 µl of #3	500µl	2.5 ng/ml
#5	500 µl of #4	500µl	1.25 ng/ml
#6	500 µl of #5	500µl	0.625 ng/ml
#7	500 µl of #6	500µl	0.312 ng/ml

Biotinylated anti leptin primary antibody were rehydrated with 100μl of 1x assay buffer, diluted to 1:400 with 100μl of 1x assay buffer, and then thoroughly mixed with vortex. After that, positive control was rehydrated with 250μl of 1x assay buffer, thoroughly mixed, and spun down. Capture antibody-coated plate was removed from its zip-lock foil pouch. Each well was washed with 300μl of 1x assay buffer, and was allowed to sit for 5 minutes. After the buffer had been discarded, the plate was inverted and blotted dry without letting well to dry before proceeding to the next step. A-1 and A-2 wells were left as blank. The prepared human leptin standard solutions were added in 100μl volume in reverse order of serial dilution into wells from B-1 and B-2 to H-1 and H-2, respectively. The assay was done in duplicate. 100μl of human leptin positive control solution was added into wells A-3 and A-4 as well as 100μl of diluted samples in duplicate were added into their designated wells as shown in Figure 9. The immunoplate was sealed with APS and was incubated at room temperature for 2 hours with orbital shaking at 300-400 rpm. APS was removed from the immunoplate and contents of the wells were completely discarded. Each well was washed for 4 times with 350μl of 1x assay buffer; after the buffer had been discarded, the plate was inverted and blotted dry. Then, 100μl of biotinylated anti-human leptin detection antibody was added into each well. The immunoplate was resealed with plate sealer incubating at room temperature for 2 hours with orbital shaking at 300-400 rpm. The SA-HRP was centrifuged at 3,000-5,000 rpm for 5 seconds, diluted with 1x assay buffer to 1:2,000, and thoroughly mixed with vortex. APS was removed from the immunoplate and contents of the wells were discarded. Each well was washed with 350μl of 1x assay buffer for 4 times; after the buffer had been discarded, the plate was inverted and blotted dry. 100μl of SA-HRP solution was added into each well; then immunoplate was resealed with APS incubating for 30 minutes at room temperature with orbital shaking at 300-400 rpm. After that, APS was removed and the immunoplate was washed and blotted dry for 4 times with 350μl of 1x assay buffer. Next, 100μl of the TMB substrate solution was added into each well and the plate was resealed with plate sealer covered with foil incubating for 30 minutes at room temperature with orbital shaking at 300-400 rpm. 100 μl of 2N HCl was added into each well to terminate reaction. The color in the well changed from blue to yellow. Finally, the immunoplate was loaded onto a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, US) and absorbance optical density (O.D.) was read at 450 nm within 20 minutes. Intra-assay coefficients

of variances were 8.955%. The standard curve was plotted with known concentrations of standard peptide on the log scale on X-axis and corresponding on the log scale O.D. on Y-axis. The standard curve shows a correlated relationship between peptide concentration and the corresponding absorbance as shown in Figure 10

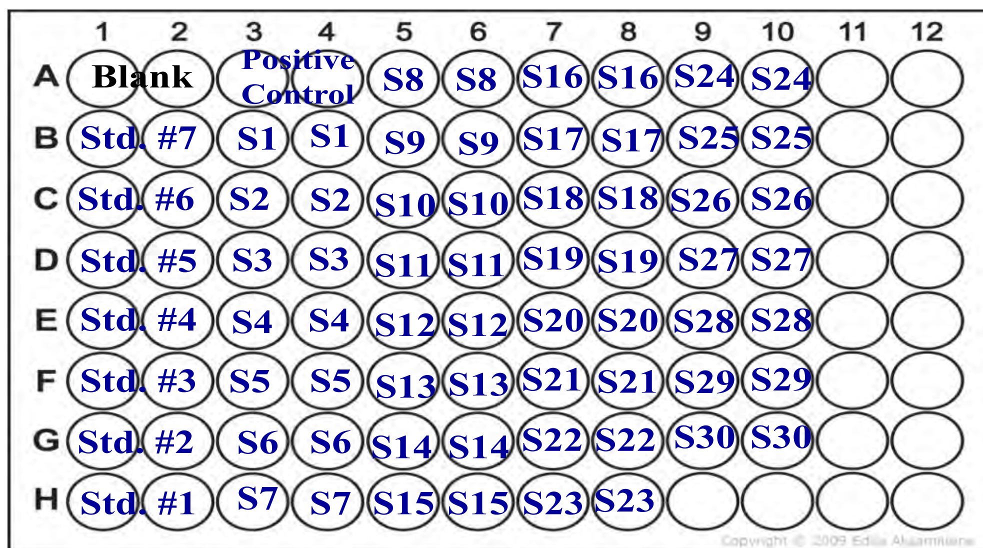


Figure 9 Assay diagram designation for serum leptin; Std. stands for peptide standard, S stands for serum.

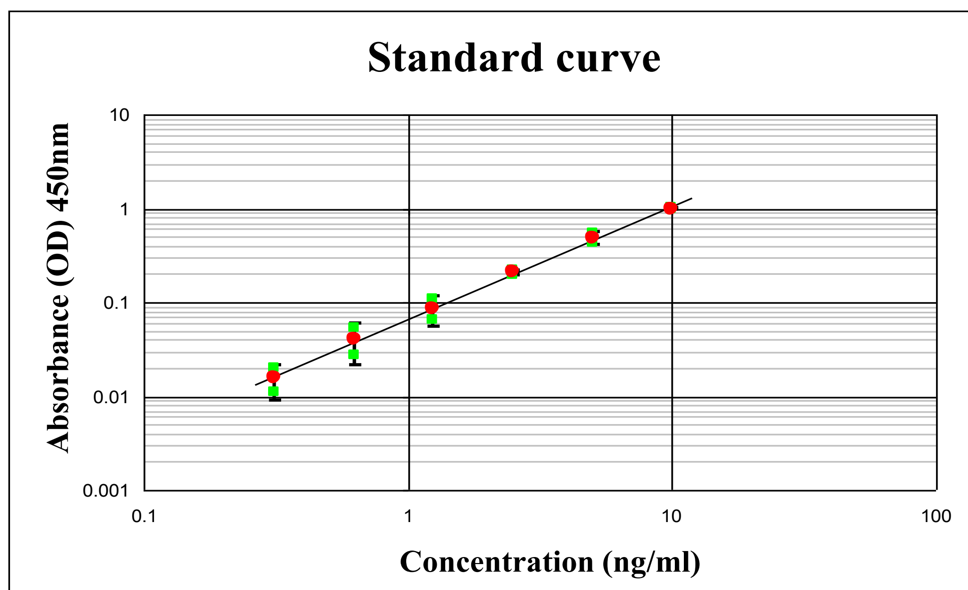


Figure 10 The standard curve of leptin

Statistical analysis

Kolomonov-Smirnov test was performed to test normality. Comparison between obese and normal weight groups was performed by Unpaired t-Test and comparison between subcutaneous and visceral adipose tissues was presented by Paired t-Test. Comparisons of data with non-normal distribution was performed with non-parametric test. Data was presented as mean ± S.E.M. Correlation coefficients was calculated using 2-tailed Pearson product-moment correlation. A p value less than 0.05 was considered as of statistical significance.

RESULTS

Clinical Parameters and Peripheral Metabolic Factors

To compare clinical parameters and peripheral metabolic factors between obese and normal weight subjects, mean age, BMI, SBP, DBP, plasma glucose, plasma insulin, and HOMA-IR were determined as shown in Table 5. Mean age (± SEM) was similar between obese and normal weight subjects which was 46.71 ± 1.38 years in obese and 43 ± 2.36 years in normal weight subjects. Mean BMI (± SEM) was 30.10 ± 1.29 kg/m^2 for obese group and 21.23 ± 0.49 kg/m^2 for normal weight subjects ($p < 0.001$). SBP, DBP, plasma insulin, and HOMA-IR were significantly greater in obese subjects than in normal weight subjects (133.54 ± 3.96 mmHg in obese subjects vs. 112.88 ± 2.15 mmHg in normal weight subjects for SBP, 74.87 ± 2.15 mmHg in obese subjects vs. 66.67 ± 2.12 mmHg in normal weight subjects for DBP, 8.13 ± 1.40 µU/ml in obese subjects vs. 3.69 ± 0.67 µU/ml in normal weight subjects for plasma insulin, and 1.64 ± 0.31 in obese subjects VS 0.68 ± 0.15 in normal weight subjects for HOMA-IR). However, there was no statistically significant difference in mean (± SEM) plasma glucose between these 2 groups which was 83.53 ± 2.00 mg/dl in obese and 84.38 ± 3.06 mg/dl in normal weight subjects.

Table 5 Distribution of subjects and peripheral metabolic factors compared between obese and normal weight patients (mean ± SEM)

Subjects/ peripheral metabolic factors	Obese	Normal weight
Number of patients	17	9
Age, years (mean ± SEM)	46.71 ± 1.38	43 ± 2.36
BMI, kg/m^2 (mean ± SEM)	30.10 ± 1.29***	21.23 ± 0.49
Systolic blood pressure, mmHg	133.54 ± 3.96**	112.88 ± 2.15
Diastolic blood pressure, mmHg	74.87 ± 2.15*	66.67 ± 2.12
Plasma glucose, mg/dl	83.53 ± 2.00	84.38 ± 3.06
Plasma insulin, μU/ml	8.13 ± 1.40*	3.69 ± 0.67
HOMA-IR	1.64 ± 0.31*	0.68 ± 0.15

Values are expressed as mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.01 compared with normal weight subjects

NPY receptor mRNA expression in adipose tissues

This study showed comparison of Y1R, Y2R, and Y5R mRNA expressions between obese and normal weight subjects and comparison between subcutaneous and visceral adipose tissues of obese, normal weight, and overall subjects.

Y1R mRNA expression in adipose tissues

Y1R mRNA expression was higher in obese group than in normal weight group in visceral adipose tissues ($p < 0.01$) but not in subcutaneous adipose tissue as shown in Figure 11A. Y1R gene expression was higher in subcutaneous than in visceral adipose tissues in obese, normal weight and overall subjects; however, significance was found only in obese and overall subjects ($p < 0.05$) as shown in Figure 11B.

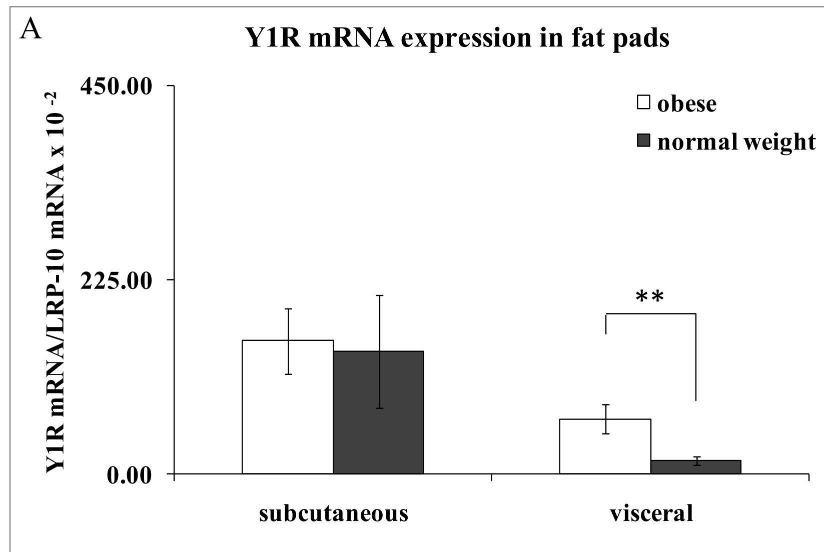
4.3.2 Y2R mRNA expression in adipose tissues

Y2R mRNA expression was greater in normal weight group than in obese group in visceral adipose tissues ($p < 0.05$) (Figure. 12A) but not in subcutaneous

adipose tissue. Y2R mRNA expression was significant higher in subcutaneous adipose tissue than in visceral adipose tissues in obese subjects whereas was not differently expressed in normal weight, and overall subjects (Figure. 12B).

4.3.3 Y5R mRNA expression in adipose tissues

Y5R mRNA expression was greater in obese group than in normal weight group in both subcutaneous ($p < 0.01$), and visceral adipose tissues ($p < 0.05$) (Figure. 13A). However, there was no statistically significant difference in Y5R mRNA between subcutaneous and visceral adipose tissue in obese, normal weight, and overall subjects (Figure. 13B).



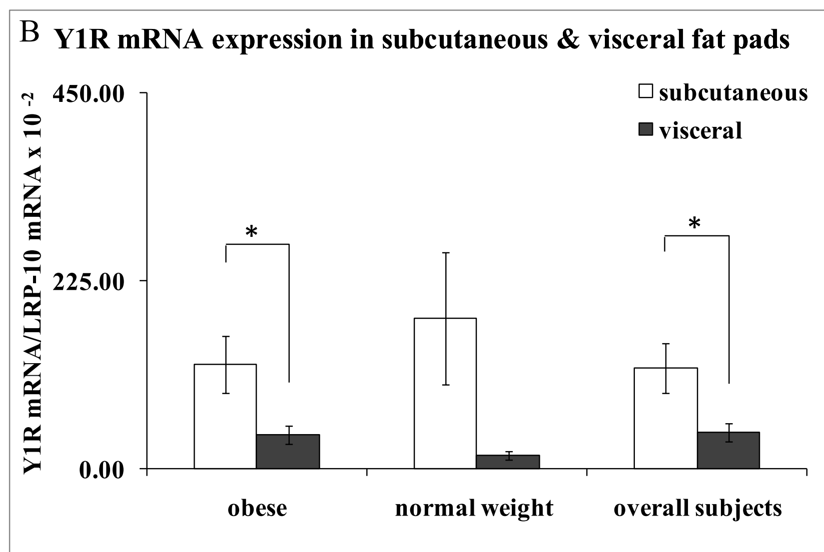


Figure 11 Mean (± SEM) Y1R mRNA expression normalized to LRP-10 (reference gene) in subcutaneous and visceral adipose tissue specimens. Panel A shows Y1R mRNA expression compared between obese and normal weight subjects in subcutaneous and visceral fat pads. Panel B shows Y1R mRNA expression compared between subcutaneous and visceral fat pads in obese, normal weight, and overall subjects. *$p<0.05$, **$p<0.01$ compared between groups.

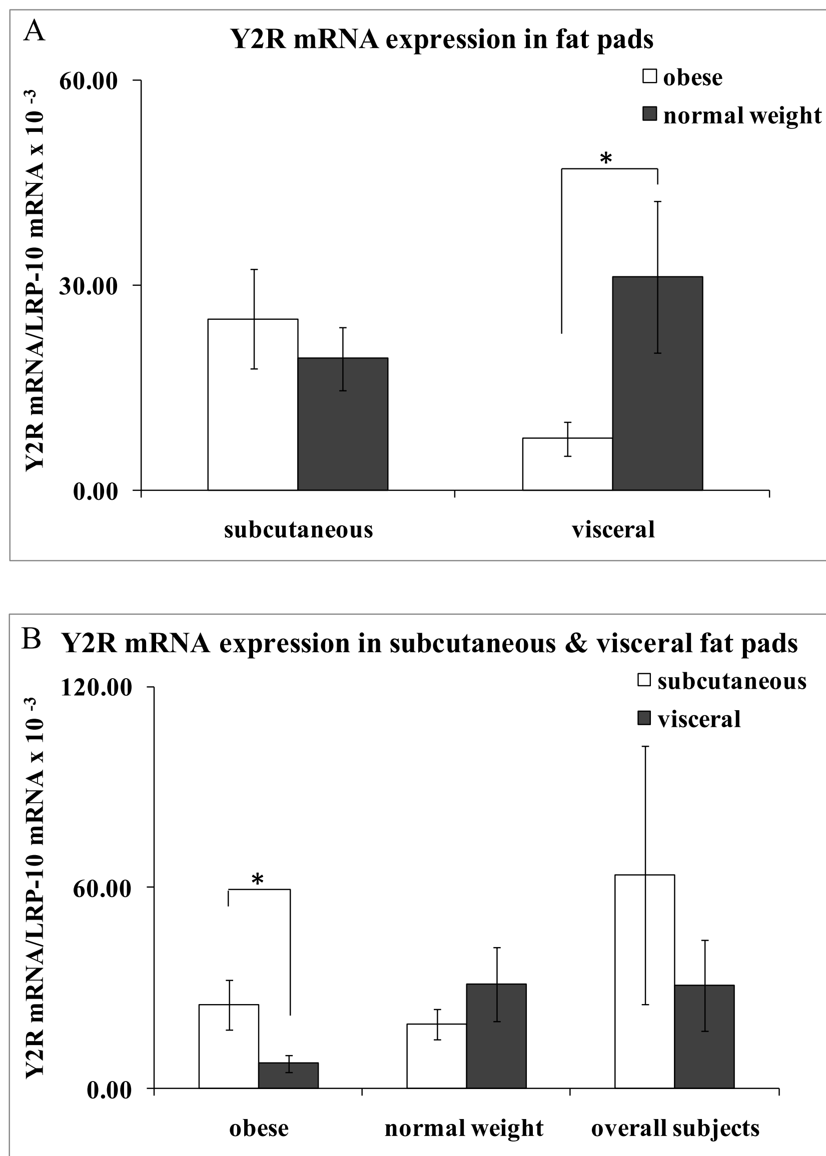


Figure 12 Mean (± SEM) Y2R mRNA expression normalized to LRP-10 (reference gene) in subcutaneous and visceral adipose tissue specimens. Panel A shows Y2R mRNA expression compared between obese and normal weight subjects in subcutaneous and visceral fat pads. Panel B presents Y2R mRNA expression compared between subcutaneous and visceral fat pads in obese, normal weight, and overall subjects. *$p<0.05$ compared between groups.

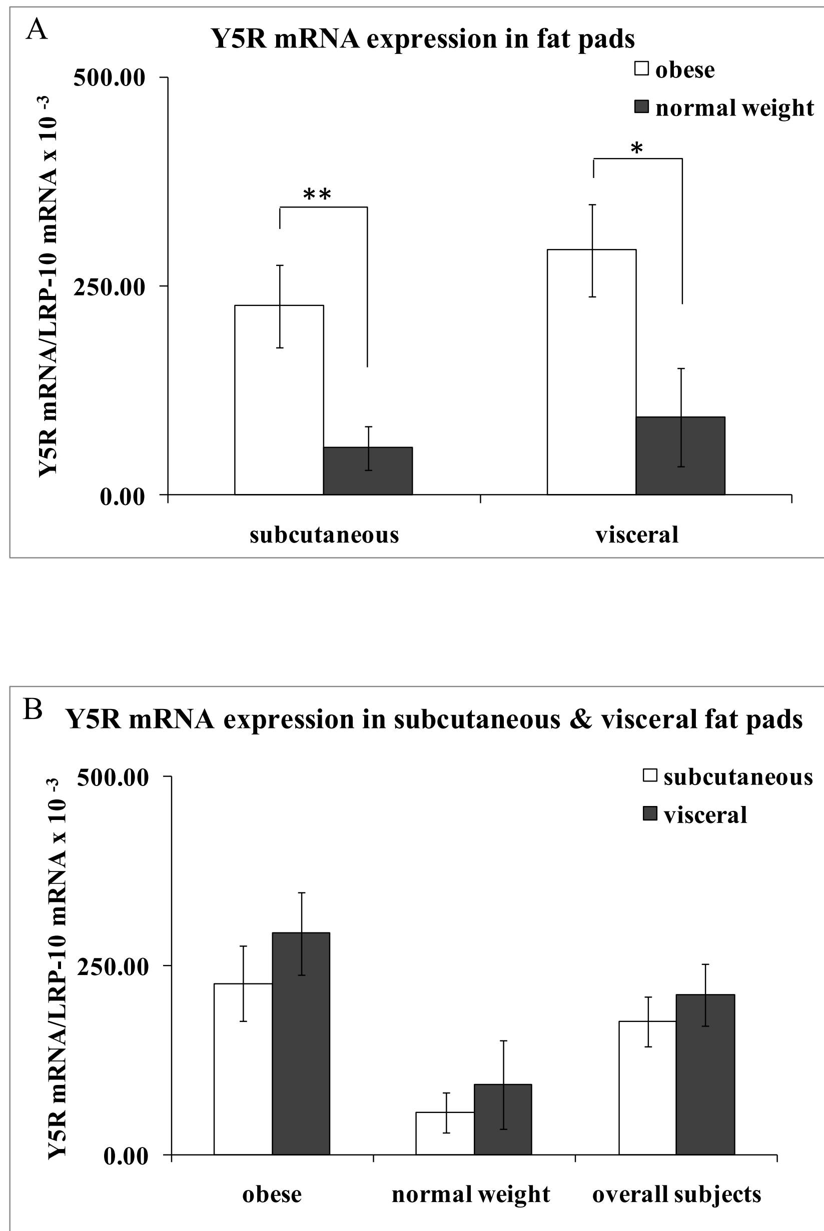


Figure 13 Mean (± SEM) Y5R mRNA expression normalized to LRP-10 (reference gene) in subcutaneous and visceral adipose tissue specimens. Panel A shows Y5R mRNA expression compared between obese and normal weight subjects in subcutaneous and visceral fat pads. Panel B shows Y5R mRNA expression compared between subcutaneous and visceral fat pads in obese, normal weight, and overall subjects. *$p<0.05$, **$p<0.01$ compared between groups.

Summary of comparisons of the gene expressions between obese and normal weight subjects

Y5R gene expressions were higher in obese subjects in both subcutaneous and visceral adipose tissues when compared with normal weight subjects. Y1R gene expression was higher in obese than normal weight subjects in visceral adipose tissue but not in subcutaneous adipose tissue. Y2R mRNA expression was greater in normal weight group than obese group in visceral adipose tissue but not in subcutaneous adipose tissue. The summary of gene expressions compared between obese and normal weight groups is shown in Table 6

Table 6 Comparisons of gene expression in adipose tissue between obese and normal weight subjects

Gene	Subcutaneous fat	Visceral fat
Y1R	⟷	↑ **obese**
Y2R	⟷	↑ **normal weight**
Y5R	↑ **obese**	↑ **obese**

Summary of comparisons of gene expressions between subcutaneous and visceral adipose tissues in obese, normal weight, and overall subjects

Y1R mRNA was higher in subcutaneous fat tissue when compared with visceral fat tissue in both obese and overall humans. Y2R mRNA was also greater in subcutaneous than visceral adipose tissue in obese group. There was no difference of Y5R mRNA expression between subcutaneous and visceral fat tissue in obese, normal weight, and overall subjects. The summary of gene expressions compared between subcutaneous and visceral fat tissue in subjects is shown in Table 7

Table 7 Comparisons of gene expression in adipose tissue between obese and normal weight subjects

Gene	Obese subjects	Normal weight subjects	Overall subjects
Y1R	↑ S	↔	↑ S
Y2R	↑ S	↔	↔
Y5R	↔	↔	↔

Significant correlations between 2 factors

The possible associations between gene expression, clinical parameters and peripheral metabolic factors were performed by Pearson correlation as shown in figure 14-18 and in Table 8 Age was positively correlated with body weight ($R = 0.522$, $p < 0.01$) and BMI ($R = 0.563$, $p < 0.01$). Body weight and BMI had positive correlations with insulin ($R = 0.639$, $p < 0.001$ and $R = 0.603$, $p < 0.001$, respectively) and HOMA-IR ($R = 0.642$, $p < 0.001$ and $R = 0.617$, $p < 0.001$, respectively). Systolic blood pressure was positively correlated with body weight ($R = 0.397$, $p < 0.05$), BMI ($R = 0.404$, $p < 0.05$), insulin ($R = 0.455$, $p < 0.05$), glucose ($R = 0.434$, $p < 0.05$) and HOMA-IR ($R = 0.509$, $p < 0.01$). A positive correlation was found between Y1R mRNA expression in subcutaneous adipose tissue and serum NPY levels ($R = 0.426$, $p < 0.05$). Y1R gene expression in visceral adipose tissue was positively correlated with body weight ($R = 0.586$, $p < 0.01$), BMI ($R = 0.611$, $p < 0.01$), waist circumference ($R = 0.474$, $p < 0.05$), hip circumference ($R = 0.483$, $p < 0.05$), insulin ($R = 0.539$, $p < 0.01$), and HOMA-IR ($R = 0.480$, $p < 0.05$). For Y2R mRNA expression, a strong positive correlation was existed between the expression in subcutaneous and visceral adipose tissues ($R = 0.932$, $p < 0.001$). Y5R mRNA expression in visceral fat tissue was positively correlated with body weight ($R = 0.415$, $p < 0.05$), BMI ($R = 0.453$, $p < 0.05$), hip circumference ($R = 0.418$, $p < 0.05$), and visceral Y1R mRNA expression ($R = 0.773$, $p < 0.001$); whereas, there was a trend to be positively correlated between Y5R expression in visceral fat with waist circumference ($R = 0.384$, $p = 0.053$), and with insulin ($R = 0.369$, $p = 0.063$). Furthermore, positive correlation was found between Y5R mRNA expression in subcutaneous adipose tissue and Y2R mRNA expression in subcutaneous adipose

tissue (R = 0.437, p < 0.05). Serum leptin levels were positively correlated with NPY mRNA expression in subcutaneous adipose tissue (R = 0.429, p < 0.05), and Y5R mRNA expression in visceral adipose tissue (R = 0.435, p < 0.05).

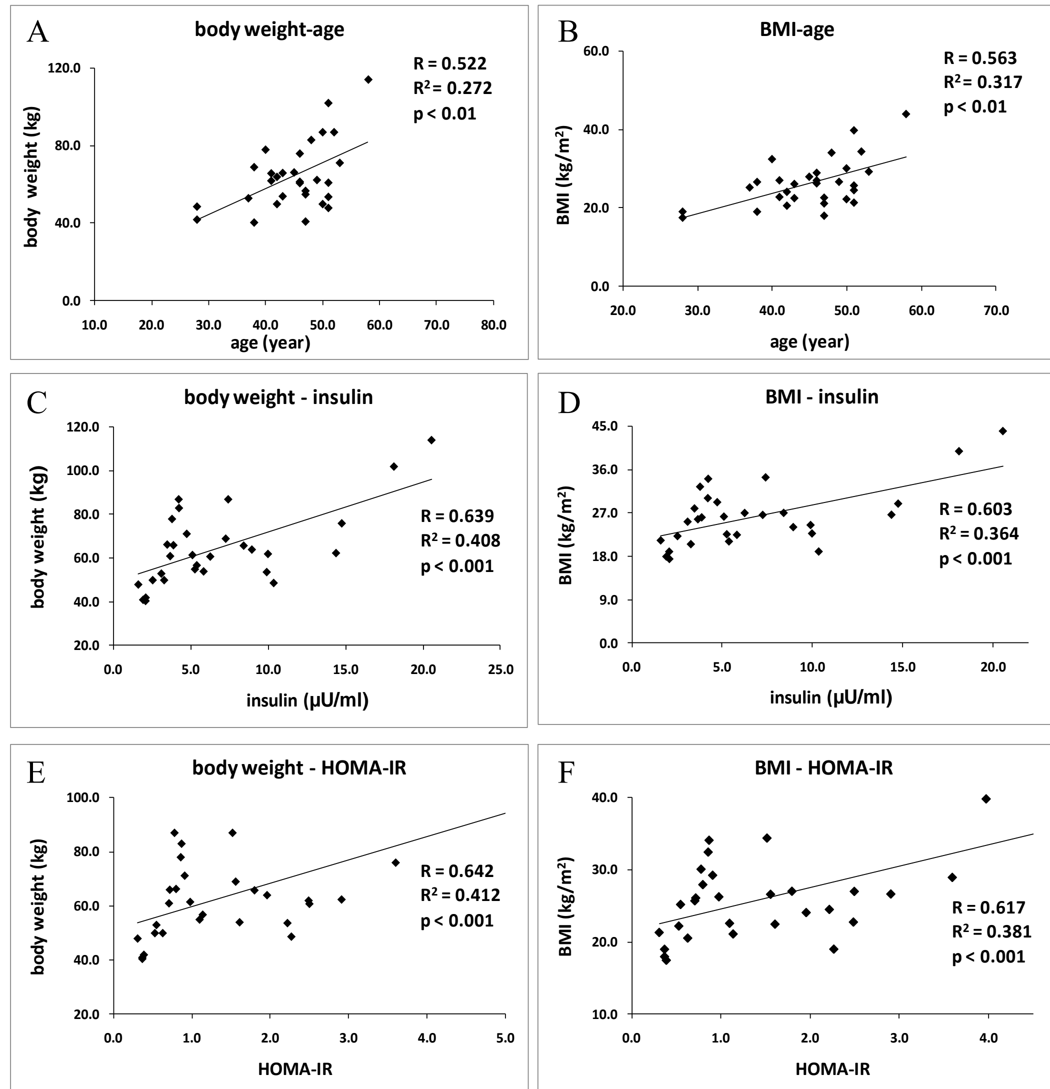


Figure 14 Significant correlations between 2 factors. Figure A-F show positive correlations of body weight or BMI with age, insulin, and HOMA-IR, respectively

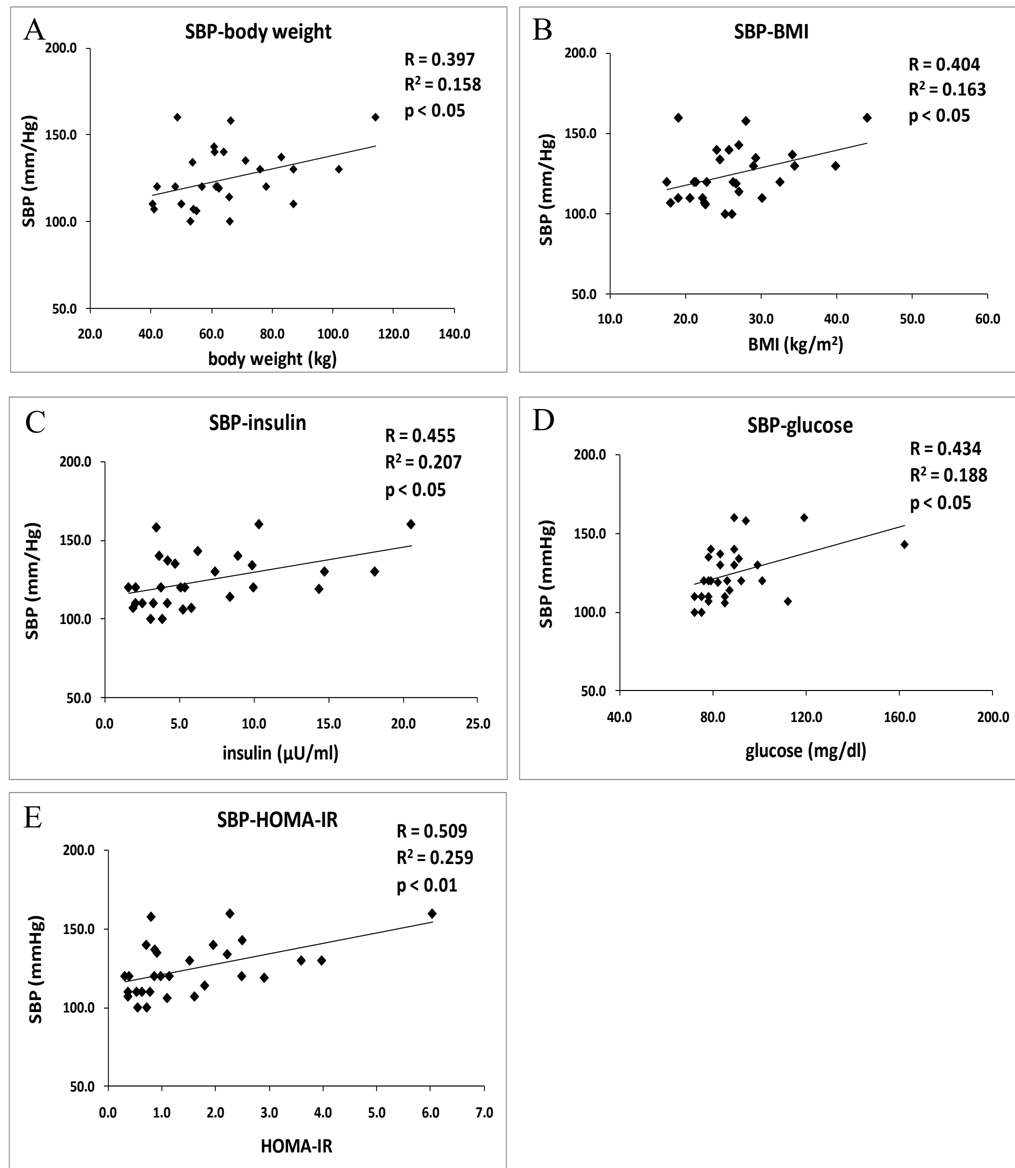


Figure 15 Significant correlations between 2 factors. Figure A-E show positive correlations of SBP with body weight, BMI, glucose, insulin, and HOMA-IR, respectively

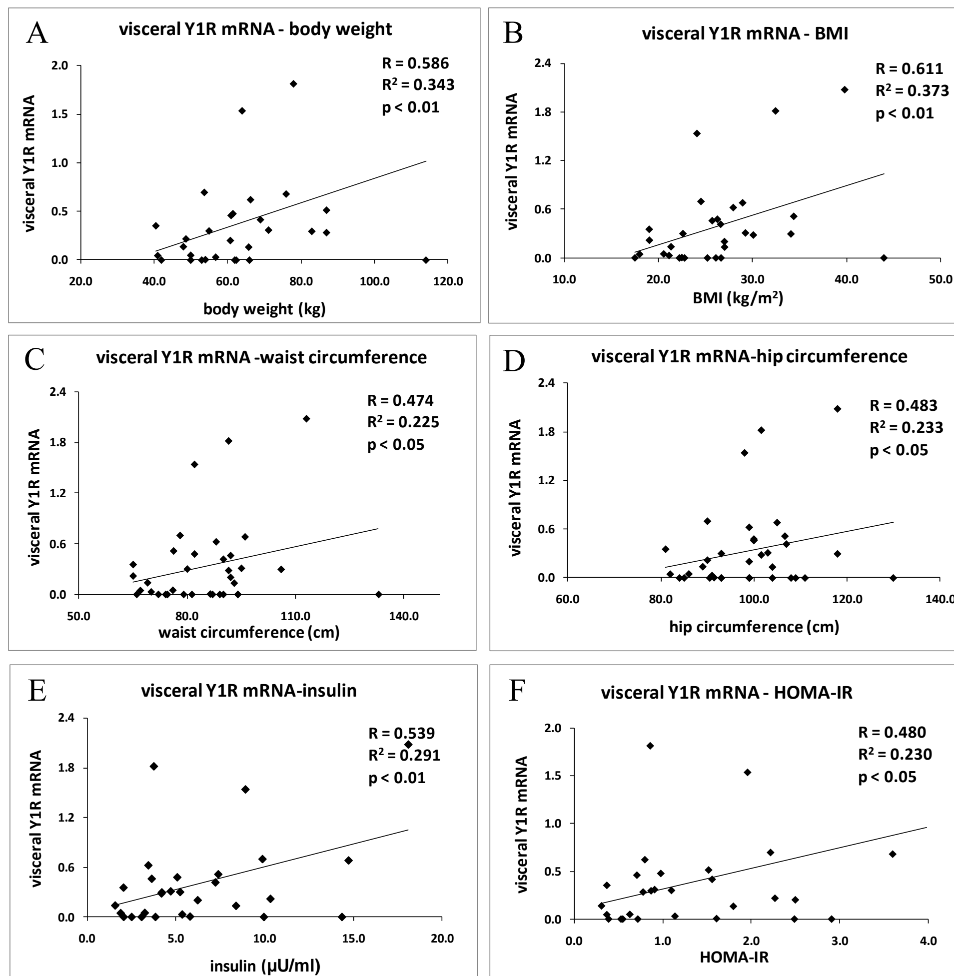


Figure 16 Significant correlations between 2 factors. Figure A-F show positive correlations of Y1R mRNA expression in visceral adipose tissue with body weight, BMI, waist and hip circumferences, insulin, and HOMA-IR, respectively

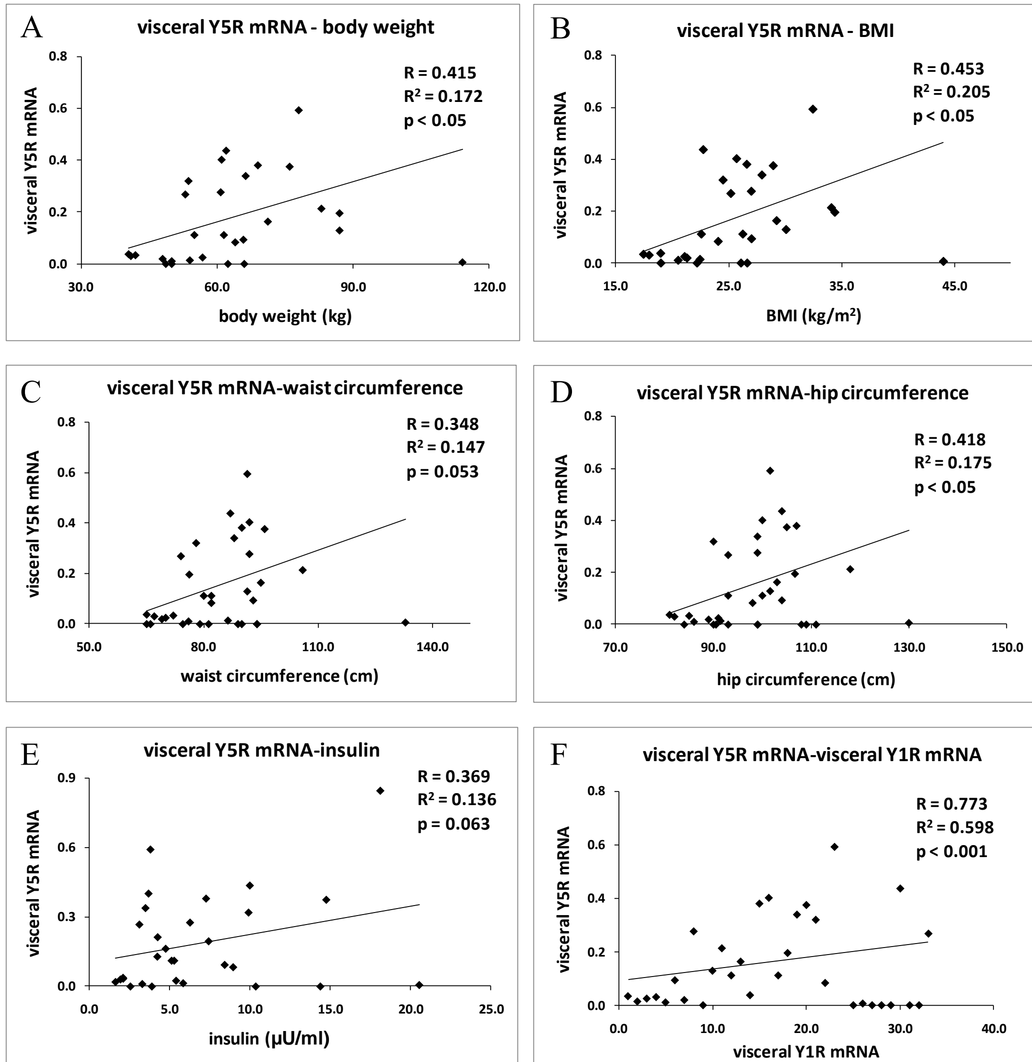


Figure 17 Significant correlations between 2 factors. Figure A-F show positive correlations of Y5R mRNA expression in visceral adipose tissue with body weight, BMI, waist and hip circumferences, insulin, and visceral Y1R mRNA, respectively

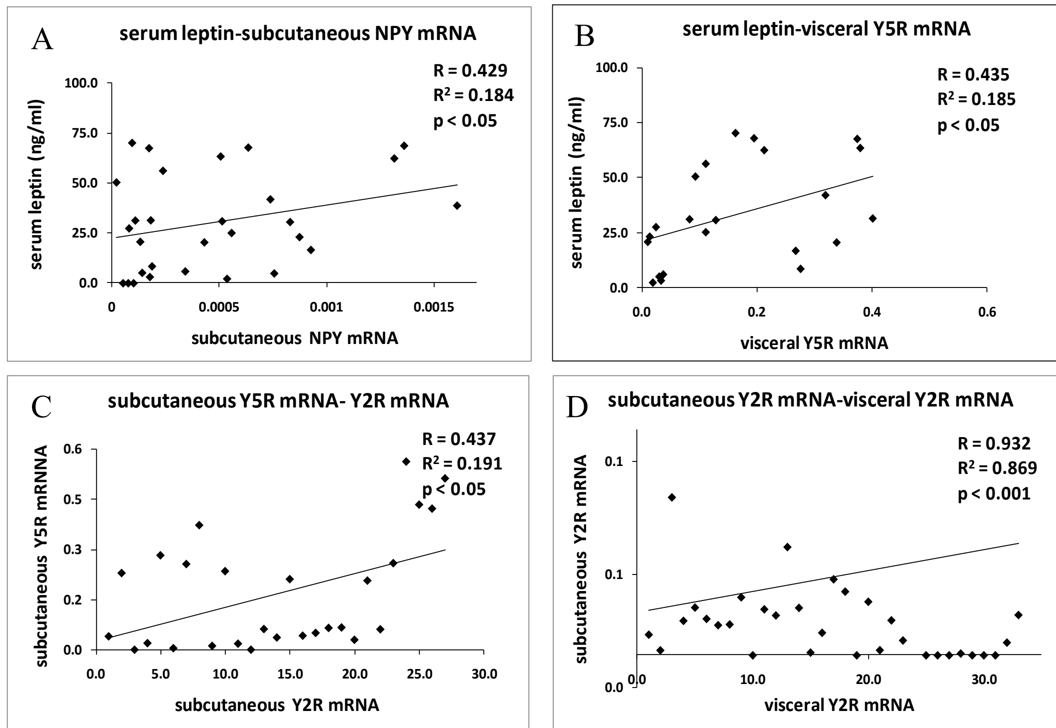


Figure 18 Significant correlations between 2 factors. Figure A and B show positive correlations of serum leptin levels with subcutaneous NPY mRNA and visceral Y5R mRNA, respectively**.** Figure C shows correlations between subcutaneous Y5R mRNA and subcutaneous Y2R mRNA. Figure D represents positive correlation between subcutaneous Y2R mRNA and visceral Y2R mRNA. Figure E shows correlation between serum NPY and subcutaneous Y1R mRNA.

Table 8 Significant correlations between 2 factors

Factors	Pearson correlation (R)	R^2	P-value
Age-body weight	0.522**	0.272	< 0.01
Age-BMI	0.563**	0.317	< 0.01
Body weight-SBP	0.397*	0.158	< 0.05
Body weight-insulin	0.639**	0.408	< 0.001
Body weight-HOMA-IR	0.642**	0.412	< 0.001
BMI-SBP	0.404*	0.163	< 0.05
BMI-insulin	0.603**	0.364	< 0.001
BMI-HOMA-IR	0.617**	0.381	< 0.001
SBP-insulin	0.455*	0.207	< 0.05
SBP-glucose	0.434*	0.188	< 0.05
SBP-HOMA-IR	0.509**	0.259	< 0.01
Visceral Y1R mRNA-Body weight	0.586**	0.343	< 0.01
Visceral Y1R mRNA-BMI	0.611**	0.373	< 0.01
Visceral Y1R mRNA-waist circumference	0.474*	0.225	< 0.05
Visceral Y1R mRNA-hip circumference	0.483*	0.233	< 0.05
Visceral Y1R mRNA-Insulin	0.539**	0.291	< 0.01
Visceral Y1R mRNA-HOMA-IR	0.480*	0.230	< 0.05
Subcut. Y2R mRNA-visceral Y2R mRNA	0.932**	0.869	< 0.001
Visceral Y5R mRNA-Body weight	0.415*	0.172	< 0.05
Visceral Y5R mRNA-BMI	0.453*	0.205	< 0.05
Visceral Y5R mRNA-hip circumference	0.418*	0.175	< 0.05
Visceral Y5R mRNA-visceralY1R mRNA	0.773**	0.598	< 0.001
Visceral Y5R mRNA-waist circumference	0.348	0.147	= 0.053
Visceral Y5R mRNA-insulin	0.369	0.136	= 0.063
Subcut. Y5R mRNA-subcut. Y2R mRNA	0.437*	0.191	< 0.05
Serum leptin- visceralY5R mRNA	0.435*	0.189	< 0.05

CHAPTER V
DISCUSSION

It is well known that human NPY mRNA and protein were identified in the CNS (17-20), especially in the hypothalamus (17-24) and NPY mRNA was detected in adipose tissues of rats (16), mice (15), and humans (13). Y1R, Y2R and Y5R mRNA are enormously expressed in human brain (24) and subcutaneous abdominal adipose tissue in human or mice (15, 41). This study focused on comparisons of NPY, Y1R, Y2R, and Y5R mRNA expression in subcutaneous and visceral adipose tissues in normal weight and obese humans and their correlations with clinical parameters and peripheral metabolic factors.

In this study, age was positively correlated with body weight and BMI indicating that aging increases proportion of fat mass possibly caused from reductions in resting metabolic rate and physical activity, as mentioned in a previous report on aging (234). Obese subjects had higher plasma insulin and HOMA-IR levels than normal weight subjects while plasma glucose was similar between these groups, indicating increased insulin resistance in the obese subjects. Although levels of insulin and HOMA-IR were still in the normal range in obese patients (2-17 µU/ml, 1.8-2.3, respectively), the 2.2 fold (120%) increase in insulin and 2.4 fold (141%) increase in HOMA-IR levels revealed higher risk of insulin resistance in the obese group. Moreover, there were positive correlations between body weight and BMI with insulin, HOMA-IR, but not glucose, confirming that increased body weight or BMI is associated with higher risk of insulin resistance. These results were similar to a previous study showing that BMI was positively correlated with insulin (R=0.50 in women, 0.56 in men) and HOMA-IR (R=0.49 in women, 0.55 in men) in South Asian women and men (235). However, this previous study (235) showed statistically significant positive correlation between BMI and glucose but the correlation coefficients were only 0.13 in women and 0.14 in men (our result was 0.265) which had no clinical significance. As a result, increased BMI might not be directly related with blood glucose. Increased insulin resistance observed in obese humans might be explained by increased proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (236), leading to reduced insulin sensitivity through long term inhibitory effects on the gene transcriptions of insulin-receptor substrate

(IRS-1), glucose transporter 4 (GLUT-4), and peroxisome proliferator-activated receptor gamma (PPAR-γ) (205, 206). Systolic blood pressure was significantly greater in obese than in normal weight subjects by 1.2 fold and mean SBP in the obese group was 133.54 mmHg which is higher than 130 mmHg (1 diagnostic criteria of metabolic syndrome criteria) (237). Moreover, SBP positively correlated with body weight and BMI which is suggestive of increased hypertension in obese humans (238). These findings are consistent with data in previous studies showing that weight gain contributed to hypertension (239-241) leading to increased cardiovascular disease risk (242, 243). In addition, SBP had positive correlations with plasma insulin, glucose and HOMA-IR implying that insulin resistance might be involved in an increased risk of hypertension. Previous studies have shown that hyperinsulinemia and/or insulin resistance was (were) (a) risk factor(s) of hypertension by increasing of sympathetic activity or intracellular Ca^{2+} leading to increased total peripheral vascular resistance (238, 244-246). Furthermore, the positive correlation between glucose and SBP was accordant with a previous study revealing that blood pressure was increased in rat treated with glucose when compare with control (247); however, the underlying mechanism was unclear. Mean DBP was also significantly greater in obese than normal weight subjects and indicative of increased metabolic risk in obese subjects; however, the levels of DBP in obese subjects were still in the normal range. As a result, DBP might be a less sensitive indicator for detection of metabolic syndrome than SBP.

Comparison between obese and normal weight subjects showed that Y1R mRNA expression was 4 fold significantly greater in obese than in normal weight subjects in visceral adipose tissue but not in subcutaneous adipose tissue. Moreover, there were positive correlations between Y1R mRNA expression in visceral adipose tissue and body weight, BMI, waist and hip circumference. These results indicated that high expression of Y1R in visceral adipose tissue might be closely associated with obesity. Previous studies showed that injection of Y1R antagonist reduced body weight (42) as well as Y1R deletion and caused a body weight reduction in male and female mice (248). It has been revealed that intracerebroventricular (icv) injected NPY significantly reduced food intake in Y1 receptor-deficient mice (163) as well as that administration of Y1R suppressed food intake in high-fat diet-fed obese mice (42). Furthermore, NPY stimulated rat preadipocyte proliferation via Y1R mediated through the activation of ERK1/2 (16), and the strong antilypolytic effect of NPY was

blocked by specific Y1R antagonists (44). As a result, Y1R expression, especially in visceral adipose tissue, might be closely related to increased obesity and fat accumulation. Our data showing comparable levels of expression of Y1R mRNA in subcutaneous tissue between obese and normal weight subjects were not similar to another publication reporting a significantly higher expression of Y1R in subcutaneous adipose tissue in obese humans when compared to the lean cohort (41). This dissimilar result might be explained by the use of different groups for comparison. In our study, we compared obese and normal weight subjects while another study (41) compared obese and lean individuals. Moreover, differences in BMI cut-off points and ethnicity might also be associated reasons. Y1R expression in visceral fat was also positively correlated with insulin levels and HOMA-IR, indicating associations between NPY action via Y1R with hyperinsulinemia and insulin resistance. It has been reported that a single dose icv administration of Y1R agonist increased plasma insulin levels at 15 minutes, 1 hour and 2 hours after injection in satiated Long-Evan rats with and without the presence of food (160) indicating that Y1R is closely related to hyperinsulinemia. A previous study reported that NPY and Y1R agonist reduced insulin induced translocation of GLUT4 from intracellular stores to cell surface in 3T3-L1 adipocytes and Y1R antagonist prevent the reduction indicating that NPY increased insulin resistance via Y1R (161). In conclusion, Y1R mRNA expression in visceral fat is closely related to obesity and metabolic syndrome.

Y1R mRNA expression was highly expressed in subcutaneous adipose tissue when compared to visceral adipose tissue in overall, obese and normal weight subjects possibly reflecting that Y1R was more highly synthesized in subcutaneous than visceral adipose tissue. Unfortunately, we didn't have enough fat tissue to confirm a protein expression study. It has been reported that Y1R protein expression indicated no significant difference between subcutaneous and omental human adipose tissues (41), but the number of subjects in this study was only 6. Additionally, microarray study showed highest expression of Y1R to be in subcutaneous adipose tissue at least 5 fold more than in other tissues (including brain, adrenal gland, and uterus) indicating that NPY mediated its action via Y1R mainly at subcutaneous adipose tissue (41); however, the expression in visceral adipose tissue was not mentioned in that study (41). Y1R mRNA expression in subcutaneous adipose tissue positively correlated with serum NPY. Moreover, serum NPY, NPY mRNA, Y1R

mRNA in subcutaneous fat tissue did not show any statistically significant correlation between body weight, BMI nor clinical parameters. These results can be interpreted that NPY action via Y1R in subcutaneous adipose tissue might be increased along with increased NPY levels in the circulation but neither obesity nor other clinical parameters.

In this study, Y2R mRNA was detected in human adipose tissue and Y2R mRNA expression was 4.1 fold significantly higher in normal weight group than obese group in visceral fat tissue but not in subcutaneous fat tissue. The result might be indicated that higher expression of Y2R mRNA in normal weight subjects in visceral adipose tissue might be involved in decreased adiposity. Previous study revealed that Y2R selective agonist (BT-48) treatment reduced ad libitum food intake in fasting mice (165) consistent with another publication showed that Y2R agonist ($PYY_{3\text{-}36}$) decreased body weight and cumulative food intake in DIO mice (45). However, chronic stress induces NPY release and upregulated Y2 expression in visceral fat in high-fat, high-sugar fed mice, resulting in abdominal obesity, inflammation, hyperlipidemia, hyperinsulinemia, glucose intolerance, hepatic steatosis, and hypertension (170). This dissimilar result might be described by the distinction of spices. Further studies are required to disclose the effect of NPY via Y2R on fat accumulation, especially in visceral adipose tissue. Y2R mRNA in subcutaneous adipose tissue was comparable between obese group and normal weight group. It has been reported that Y2R mRNA expression in subcutaneous abdominal tissue was markedly up-regulated in genetically leptin deficient obese (ob/ob) mice when compared with control C57BL/6J mice (15). Additionally, fat accumulation and metabolic complications can be prevented or reversed by pharmacological Y2R inhibition at local intra-fat or adenoviral Y2R knock-down in mice (170). Previous study showed that NPY stimulated fat growth via Y2R in human and mice preadipocytes (15) so the action of NPY via Y2R in subcutaneous fat might be involved in adipogenesis. It has appeared that peripheral function of Y2R in fat accumulation was different between subcutaneous and visceral adipose tissue and this issue needs to be further investigated.

Y2R mRNA expression was 3.3 fold higher in subcutaneous adipose tissue when compared to visceral adipose tissue in obese but not in other groups indicating that Y2R in obese humans was more capable synthesized in subcutaneous than in visceral fat tissue. Previous study in human showed that fat cell volume of

obese subjects was greater than non-obese subjects (249) with adipocyte size in subcutaneous being larger than adipocyte size in omental adipocyte (194). Thus, it is possible that Y2R was more synthesized in subcutaneous than visceral adipose tissue resulting from ability of large adipocyte. Furthermore, this study found a strong positive correlation between Y2R mRNA expression in subcutaneous and visceral adipose tissue suggesting a concordant action of this receptor in 2 depots.

Y5R mRNA expression was significantly higher in obese group than in normal weight group in both subcutaneous and visceral fat tissue with increased levels of expression in obese people by 4 fold in subcutaneous and by 3.1 fold in visceral adipose tissues implying Y5R might be involved in increased adiposity. Moreover, Y5R mRNA expression in visceral adipose tissue had significantly positive correlations with body weight, BMI, and hip circumference, and serum leptin levels and it had a trend of positive correlation with waist circumference ($R = 0.384$, $p = 0.053$). These results indicated that high expression of Y5R mRNA, especially in visceral adipose tissue, might be closely related with obesity and might be predicted by measurements of body weight, BMI, waist circumference, and hip circumference. It has been revealed that oral treatment of Y5R antagonist was significantly decreased fat pad weight in DIO mice and reduced adipose cell size in both obese and lean mice by 2 fold reduction in obese group when compared with lean group (250). Previous study in male satiated Long-Evans rats, icv injection of Y5 selective agonist D-[Trp^{32}]-NPY increased food intake, reduced oxygen consumption and energy expenditure (48). In mice, chronic oral administration of a selective Y5 antagonist suppressed the food intake and fat accumulation induced by NPY stimulated food intake (172) or by treatment with moderately high fat diet (MHF) (250) indicating that long term effect of the Y5 receptor stimulation leads to hyperphagia and obesity. As a result, Y5R expression, especially in visceral adipose tissue, might be closely related to increased adiposity. Y5R mRNA expression in visceral adipose tissue had a trend without statistically significant correlation with plasma insulin levels ($R = 0.369$, $p = 0.063$). It has been revealed that selective activation of Y5R induced hyperphagia and increased plasma insulin after 1 hour of injection in Long Evans rats (160). Previous study showed that Y5R antagonists at 30 and 100 mg/kg decreased plasma insulin levels in DIO mice (250) consistent with decreased plasma insulin in Y5R antagonist treated group when compared to vehicle treated group (251). These evidences might be indicated an association between NPY action through Y5R with hyperinsulinemia.

Moreover, a strong positive correlation between Y5R mRNA and Y1R mRNA expression in visceral adipose tissue was found. Both Y1R and Y5R expression in visceral adipose tissue had positive correlation with body weight, BMI, and waist and hip circumference indicating that there was a similar trend for both receptors involved in regulation of obesity. Previous study showed synergistic interaction between the Y1R and Y5R in increasing food intake and body weight (42). In addition, spontaneous and fasting-induced food intake at 18 or 34 weeks of age was reduced in germline Y1Y5 receptor double knockout ($Y1Y5^{-/-}$) male mice and body weight regain was delayed at 18 weeks of age after fasting in both $Y1Y5^{-/-}$ male and female mice when compared to wild type mice (252). Collectively, Y1R and Y5R expressions in visceral adipose tissue were strongly related to each other and levels of adiposity.

Y5R mRNA expression was not differently expressed in subcutaneous adipose tissue and visceral adipose tissue in overall, obese and normal weight subjects possibly reflecting that Y5R was equally synthesized in subcutaneous and visceral adipose tissue. The result from our study was consistent with previous study that Y5R protein expression was not differently expressed between in abdominal subcutaneous and in omental adipose tissues (41); however, our study couldn't present the comparison of protein expression levels because of the insufficient sample. In addition, there was a positive correlation between Y5R mRNA expression and Y2R mRNA in subcutaneous adipose tissue. This finding might be a coincidence or could be interpreted that expression of these two receptors in subcutaneous adipose tissue might be regulated in the same way.

In conclusion, this study revealed comparisons of NPY, Y1R, Y2R, and Y5R expressions in subcutaneous and visceral adipose tissue between obese and normal weight subjects. NPY and Y5R mRNA expressions were significantly higher in obese than normal weight in both subcutaneous and visceral adipose tissues. In visceral adipose tissue, Y1R mRNA expression was greater but Y2R was lower in obese than in normal weight group. However, in subcutaneous fat tissue, there was no statistically significant difference of Y1R and Y2R between obese and normal weight subjects. Further investigation about blocking Y5R in both subcutaneous and visceral adipose tissues as well as blocking Y1R and activating Y2R in visceral fat may reveal new strategies for adiposity reduction. Moreover, there was a strongly positive correlation between Y1R mRNA and Y5R mRNA in visceral adipose tissue. Y1R and

in visceral adipose tissue had positive correlations with body weight, BMI, waist and hip circumferences, plasma insulin, and HOMA-IR and Y5R mRNA in visceral fat positively correlated with body weight, BMI, hip and waist circumferences, and serum leptin. Thus, clinical parameters can be indicators of Y1R and Y5R mRNA expressions in visceral adipose tissue which are closely related with adiposity and risks of metabolic syndrome.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ
   ได้ submit ผลงานกับวารสาร Regulartory peptides, second revision ดังเอกสารแนบ
2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงวิชาการ ได้พัฒนาการเรียนการสอนและมีนักศึกษาปริญญาโทจบการศึกษาจากผลงานวิจัยดังกล่าว
3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
   ได้เผยแพร่โปสเตอร์ในงานประชุมวิชาการ ดังเอกสารแนบ

Poster Presentation

- Sitticharoon C., Chatree S., Churintarapan M., “NPY receptor mRNA expressions in subcutaneous and visceral adipose tissues of normal weight and obese humans”

on “The international conference in Medicine and Public Health 2012 (ICMPH2012) on the occasion of the 150th anniversary of the birth of Queen Sri Savarindira, National conference “Multidisciplinary care towards healthy Thai society” and International conference “From Royal initiatives towards Healthy Thai society”, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand,17th-21st September, 2012

Elsevier Editorial System(tm) for Regulatory Peptides
Manuscript Draft

Manuscript Number: REGPEP-D-12-00120R2

Title: Expressions of neuropeptide Y and Y1 receptor in subcutaneous and visceral fat tissues in normal weight and obese humans and their correlation with clinical parameters and peripheral metabolic factors

Article Type: Full Length Article

Keywords: NPY, Y1R, adipose tissue, obesity, metabolic syndrome

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Abstract: Recently, Neuropeptide Y (NPY) and Y1 receptor (Y1R) were found to be expressed and synthesized in adipose tissue. This study aimed to compare NPY and Y1R mRNA expression in subcutaneous and visceral fat tissues as well as serum NPY in normal weight and obese humans and their correlations with clinical parameters and peripheral metabolic factors. We demonstrate that NPY mRNA expression was higher in obese than in normal weight humans ($p<0.05$) in both subcutaneous and visceral adipose tissues and was significantly greater in visceral when compared with subcutaneous fat in overall subjects ($p<0.01$), obese ($p<0.05$) and normal weight humans ($p<0.05$). Y1R mRNA expression was higher in obese than normal weight subjects in visceral ($p<0.01$) but not in subcutaneous adipose tissue and was statistically greater in subcutaneous when compared to visceral adipose tissues in obese ($p<0.05$) and overall subjects ($p<0.05$). Serum NPY was higher in obese than normal weight groups ($p<0.05$). Obese subjects showed significantly greater levels of systolic blood pressure (SBP) ($p<0.01$), diastolic blood pressure (DBP) ($p<0.05$), plasma insulin ($p<0.05$) and HOMA-IR ($p<0.05$) when compared with normal weight subjects. Additionally, Y1R mRNA expression in visceral adipose tissue was positively correlated with body weight ($R=0.586$), BMI ($R=0.611$), waist ($R=0.474$) and hip ($R=0.483$) circumferences, insulin levels ($R=0.539$), and HOMA-IR ($R=0.480$). As the result, Y1R expression in visceral adipose tissue might be an indicator of increased risk of metabolic syndrome. Further studies about blocking specific Y1R may propose strategies for risk reduction in metabolic syndrome and prevention or treatment of obesity.

Cover Letter

Expressions of neuropeptide Y and Y1 receptor in subcutaneous and visceral fat tissues in normal weight and obese humans and their correlation with clinical parameters and peripheral metabolic factors

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***Highlights (for review)**

This article, entitled “Expressions of neuropeptide Y and Y1 receptor in subcutaneous and visceral fat tissues in normal weight and obese humans and their correlation with clinical parameters and peripheral metabolic factors”, provides novel knowledge regarding different NPY and Y1R expression and important clinical parameters found in obese and normal weight subjects. From this study, higher NPY mRNA expression in adipose tissue especially in visceral adipose tissue as well as higher serum NPY in obese people indicates that NPY might be associated with increased obesity. Furthermore, a strong negative correlation between NPY expression in visceral adipose tissue and birth weight might indicate that low birth weight is related to increased central obesity and metabolic syndrome. As a result birth weight might be an indicator for prediction of visceral NPY mRNA expression in adulthood.

Y1R mRNA expression was 4 fold significantly greater in obese than in normal weight subjects in visceral adipose tissue but not in subcutaneous adipose tissue. Moreover, there were positive correlations between Y1R mRNA expression in visceral adipose tissue and body weight, BMI, waist and hip circumference. These results indicated that high expression of Y1R in visceral adipose tissue might be closely associated with obesity. Y1R expression in visceral fat was also positively correlated with insulin levels and HOMA-IR, indicating associations between NPY action via Y1R with hyperinsulinemia and insulin resistance. Further investigation about the blocking Y1 receptor subtype, especially on adipose tissue, may reveal strategies for risk reduction in metabolic syndrome and prevention or treatment of obesity.

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Expressions of neuropeptide Y and Y1 receptor in subcutaneous and visceral fat tissues in normal weight and obese humans and their correlation with clinical parameters and peripheral metabolic factors

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ABSTRACT

Recently, Neuropeptide Y (NPY) and Y1 receptor (Y1R) were found to be expressed and synthesized in adipose tissue. This study aimed to compare NPY and Y1R mRNA expression in subcutaneous and visceral fat tissues as well as serum NPY in normal weight and obese humans and their correlations with clinical parameters and peripheral metabolic factors. We demonstrate that NPY mRNA expression was higher in obese than in normal weight humans ($p<0.05$) in both subcutaneous and visceral adipose tissues and was significantly greater in visceral when compared with subcutaneous fat in overall subjects ($p<0.01$), obese ($p<0.05$) and normal weight humans ($p<0.05$). Y1R mRNA expression was higher in obese than normal weight subjects in visceral ($p<0.01$) but not in subcutaneous adipose tissue and was statistically greater in subcutaneous when compared to visceral adipose tissues in obese ($p<0.05$) and overall subjects ($p<0.05$). Serum NPY was higher in obese than normal weight groups ($p<0.05$). Obese subjects showed significantly greater levels of systolic blood pressure (SBP) ($p<0.01$), diastolic blood pressure (DBP) ($p<0.05$), plasma insulin ($p<0.05$) and HOMA-IR ($p<0.05$) when compared with normal weight subjects. Additionally, Y1R mRNA expression in visceral adipose tissue was positively correlated with body weight ($R=0.586$), BMI ($R=0.611$), waist ($R=0.474$) and hip ($R=0.483$) circumferences, insulin levels ($R=0.539$), and HOMA-IR ($R=0.480$). As the result, Y1R expression in visceral adipose tissue might be an indicator of increased risk of metabolic syndrome. Further studies about blocking specific Y1R may propose strategies for risk reduction in metabolic syndrome and prevention or treatment of obesity.

Introduction

Obesity has become a public health problem throughout the world with 475 million adults being considered obese (data for 2010) [1] as define by body mass index (BMI) over 30 kg/m^2 [1, 2]. For the Asian population, BMI more than 25 are identified as obese, 23-24.9 as overweight, 18.5-22.9 as normal range and less than 18.5 as underweight [3]. It is well-known that obesity is closely associated with hyperinsulinemia [4, 5] and metabolic disorders [6]; these have high levels of some indicators such as Homeostatic Model for Assessment of Insulin Resistance (HOMA-IR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) [7, 8], leading to increased incidence rates of non-insulin-dependent diabetes mellitus (NIDDM), cardiovascular diseases and hypertension [2, 9, 10]. Major complex factors involved in obesity are appetite regulation and control of energy metabolism [11]. Neuropeptide Y (NPY), a 36 amino acid peptide [12], is the most potent orexigenic peptide in the brain [13] and is abundantly expressed in the arcuate nucleus (ARC) of the hypothalamus [14], cerebral cortex, hippocampus, amygdala and limbic system [14-26]. Central NPY administration induces weight gain and adiposity [27-30] by increasing appetite and vagal activity as well as suppressing the thermogenic mechanism and sympathetic outflow [31-34]. NPY is also expressed in peripheral tissues including adrenal medulla [35], sympathetic ganglia and tissues receiving dense sympathetic innervations [36-40]. Peripheral functions of NPY include regulation of angiogenesis, vasoconstriction, mood regulation and fertility [41]. Furthermore, NPY expression is also found in subcutaneous and visceral adipose tissues in humans [42, 43], rats and mice [44, 45] and NPY protein expression has been detected in human adipocytes, abdominal subcutaneous, omental and thigh adipose tissues with expression in abdominal subcutaneous adipose tissue being twofold higher than either paired omental or thigh adipose tissues [42]. Previous studies showed that expressions of many adipokines are differently expressed between obese and control subjects in subcutaneous and

visceral adipose tissues. Visceral NPY mRNA expression was higher in leptin receptor-deficient obese Zucker rats than in lean rats [45] which is consistent with up-regulation of NPY mRNA in subcutaneous abdominal fat of obese leptin-deficient (ob/ob) mice when compared with controls [44]. NPY mediates its effect via NPY receptors, Y1-Y7 [46, 47], with Y1 receptor (Y1R), Y2 receptor (Y2R), Y4 receptor (Y4R), and Y5 receptor (Y5R) presented in humans [48]. Y1R [49], Y2R [44], and Y5R [49] are expressed on adipose tissue, so NPY can act on adipose tissue in endocrine, paracrine or autocrine fashion. Y1R was identified centrally in the hypothalamus and whole brain of rats [50]and humans [49, 50] and peripherally in adipocyte of rats [45], as well as uterus, skin, and subcutaneous adipose tissue of humans [49]. The potential functions of Y1R were demonstrated via stimulatory effect of NPY on hyperphagia, vasoconstriction [48] and proliferation of primary cultures of rat preadipocytes [45]. Previous study in mice also showed that administration of Y1 receptor antagonist reduced body weight and food intake [51]. As a result, NPY and Y1 receptor activation leads to increased adiposity and their expressions on adipose tissues might be different between obese and normal weight humans. This study aimed to compare NPY and Y1R mRNA expression in subcutaneous and visceral fat tissues in normal weight and obese humans and their correlation with clinical parameters and peripheral metabolic factors.

Materials and Methods

Subjects

The study protocol was approved by the Siriraj Institutional Review Board (si533/2009) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. All subjects gave informed consent prior to the study. All 30 Thai female patients who underwent abdominal surgery were recruited and divided into 2 groups, which were obese (n=17) (BMI > 25 kg/m^2) and normal weight (n=9) (BMI 18.5 – 22.9 kg/m^2). The other 4 subjects were

overweight or lean and were recruited for correlation analysis. Subjects on endocrine therapy (e.g. steroids, hormone replacement therapy, thyroxine), and those who were pregnant, lactating, undergoing traumatic operations, had malignant diseases, or who underwent operations related to endocrine diseases and severe abdominal inflammation were excluded.

In this study, male subjects could not be recruited because most of the male patients who underwent open abdominal surgery were cancer or emergency operations which fell into the exclusion criteria. Other studies collecting visceral adipose tissue from open abdominal surgery were also done in female [42, 49]. Phase of menstrual cycle of female subjects recruited in this study couldn't be controlled because most of the subjects had myoma uteri and were presenting with irregular menstruation.

Tissue and blood collection

Blood was collected during the fasting state before operation and abdominal subcutaneous and omental (visceral) adipose tissues were collected during operation. Four to five pieces of 0.5 cm of each type of adipose tissues, which are abdominal subcutaneous and visceral adipose tissues, were collected. For each subject, both subcutaneous and visceral adipose tissues were collected. Adipose tissues were immediately snap-frozen in liquid nitrogen and stored at -80 ˚C until analysis.

Analysis of gene expression of NPY and Y1R in adipose tissues

The total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. About 1 μg of total RNA was reverse transcribed to complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-RAD, Hercules, California, USA). In order to quantify NPY and Y1R mRNA expression, real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out using the reagents and protocol contained in the VeriQuest SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, California, USA). Low density lipoprotein receptor-related protein 10 (LRP-10) was

used as a reference gene because it is the most stably expressed gene in human adipose tissue [52]. Primer sequences were designed with the exon to exon sequences and were blasted to confirm primer specificity by the authors using published nucleotide sequences from PubMed database as follows:

NPY-forward: 5'-CCAGGCAGAGATATGGAAAACGA-3'

NPY-reverse: 5'-GGTCTTCAAGCCGAGTTCTGGG-3'

Y1R-forward: 5'-ATCATGCTGCTCTCCATTGTGGT-3'

Y1R-reverse: 5'-GTTGAAGAAGAACTGCAAGTCTCTCT-3'

LRP-10-forward: 5'-GATGGAGGCTGAGATTGTGCA-3'

LRP-10-reverse: 5'- TGGAGTCATATCCTGGCGTAAG-3'

NPY product size was 102 base pairs (bp), Y1R product size was 222 base pairs (bp), and LRP-10 product size was 169 bp. The PCR amplification was performed under the following conditions: Taq DNA polymerase activation at 95 ºC for 10 min, 40 cycles of DNA denaturing at 95 ºC for 15 sec, annealing at 57 ºC for 60 sec and extension at 72 ºC for 30 sec. For every RT reaction, no template control (NTC) was performed as negative controls and human brain and placenta tissues were used as positive controls. The actual RT-PCR product size was proven using DNA ladder (BioLabs Inc, Ipswich, Massachusetts, England) by electrophoresis. The hallmarks of a real-time PCR assay optimization include 1) linear standard curve (R^2 > 0.98), 2) high amplification efficiency (90-105%), and 3) consistency across replicate reactions [53]. The efficiency of each primer was tested by amplification of real-time PCR products in 5 dilutions of cDNA. For our study, the R^2 value of linear standard curve was 0.9969 and the percentage of efficiency was 91.80213 which was consistent across duplicate reactions. Thus, our real-time PCR assay was accepted as a robust, reproducible assay. As a result, the $2^{-\Delta CT}$ method was applied as a comparative method of quantification.

Hormonal assay

Both plasma glucose and insulin were analyzed by the central laboratory of Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand by immunoturbidimetric assay (Hitachi, Chiyoda, Tokyo, Japan) for plasma glucose and by electrochemiluminescent immunoassay (Lincoln, Madera County, California, USA) for plasma insulin. Fasting plasma glucose and insulin levels can be applied to determine levels of insulin resistance using the method of the HOMA-IR which is calculated by multiplication of fasting glucose (mg/dl) and fasting insulin (µU/ml) divided by 405. HOMA-IR levels shown in non-diabetic populations from the Middle East and Southeast Asia were 1.8-2.3 [54, 55]; the HOMA-IR > 2.3 was considered as insulin resistance in this study.

Analysis of serum NPY

Fasting blood sample was balanced weight and separated phase by centrifugation at 5,000 rpm, 4 °C for 15 min. Serum sample was stored at -80 °C until analysis. NPY serum levels were assayed by a commercial enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Burlingame, California, USA). The range of NPY detection was 0-100 ng/ml and the minimum detectable concentration was 0.14 ng/ml. The absorbance O.D. was read at 450 nm by Synergy HT Multi-Detection Microplate Readers (BioTek Instruments, Inc., Winooski, VT, U.S.). Intra-assay coefficient of variance was 10.1%.

Immunohistochemistry for NPY

The adipose tissue samples were paraffin embedded, were sectioned at 20 µm thickness using a microtome (Global Medical Instrumentation Inc, Ramsey, MN, U.S.) and mounted on hydrophilic surface Twin-Mark microscope slides (Citotest Labware Manufacturing, Nanjing, Jiangsu, China). The immunohistochemistry was carried out as the published paraffin

protocol from Abcam®. Briefly, the slides were blocked with 1% BSA (Bovine serum albumin) in TBS (Tris-buffered saline) solution for 2 hrs and incubated with monoclonal antibody directed against NPY (1:300; Abnova, Neihu, Taipei, Taiwan) overnight at 4 °C. The goat anti-mouse immunoglobulin (1:20,000; Abnova, Neihu, Taipei, Taiwan) was used as secondary antibody tagged with Fluorescein isothiocyanate (FITC) and bisBenzimide Hoechst 33258 (Sigma, St Louis, MO) was used for nuclear counterstain at a final concentration of 2.5 μg/ml. Negative controls were presented by omission of primary antibody.

Statistics

Data were presented as mean ± standard deviation (SD) as SD suggests the variability among sample observation whereas a standard error of the mean predicts the variability among theoretical sample means [56]. Comparisons between obese and normal weight groups were performed by unpaired t-Test. Comparisons between subcutaneous and visceral adipose tissues were made by paired t-Test. Correlation coefficients were calculated using 2-tailed Pearson product-moment correlation method. A multivariate linear regression model was built by stepwise analysis, incorporating the variables that had shown significant correlation. The existence of collinearity among the variables included in the study was ruled out. A p value less than 0.05 was considered as of statistical significance.

Results

Clinical Parameters

Mean age (± SD) was similar between obese and normal weight subjects. This was 46.71 ± 5.68 years in obese and 43 ± 7.07 years in normal weight subjects. Mean BMI (± SD) was 30.10 ± 5.31 kg/m^2 for the obese group and 21.23 ± 1.46 kg/m^2 for normal weight subjects (p

< 0.001). Mean (± SD) levels of many clinical parameters were significantly greater in obese subjects than in normal weight subjects, including SBP (133.54 ± 14.26 mmHg in obese subjects vs. 112.88 ± 6.08 mmHg in normal weight subjects) (Table 1), DBP (74.87 ± 8.33 mmHg in obese subjects vs. 66.67 ± 5.20 mmHg in normal weight subjects) (Table 1), plasma insulin (8.13 ± 5.61 μU/ml in obese subjects vs. 3.69 ± 1.75 μU/ml in normal weight subjects) (Table 1) and HOMA-IR (1.64 ± 1.14 in obese subjects VS 0.68 ± 0.36 in normal weight subjects) (Table 1). However, there was no statistically significant difference in mean (± SD) plasma glucose between these 2 groups which was 83.53 ± 7.74 mg/dl in obese and 84.38 ± 8.65 mg/dl in normal weight subjects (Table 1).

NPY and Y1R gene expression in adipose tissue

NPY mRNA expression was higher in the obese group than in the normal weight group ($p < 0.05$) in both subcutaneous and visceral adipose tissues (Fig. 1B). Furthermore, the expression was significantly higher in visceral than in subcutaneous adipose tissues in subjects overall ($p < 0.01$), in the obese ($p < 0.05$), and in normal weight humans ($p < 0.05$) (Fig. 1C). Y1R mRNA was detected in human subcutaneous and visceral adipose tissues and the expression was greater in obese subjects than in normal weight subjects (Fig. 1D; $p < 0.01$) in visceral adipose tissue but not in subcutaneous adipose tissue (Fig. 1D). Y1R gene expression was higher in subcutaneous than in visceral adipose tissues in obese, normal weight and overall subjects; however, significance was found only in obese and overall subjects ($p < 0.05$) (Fig. 1E).

Serum NPY levels

Mean serum NPY levels (± SD) were higher in obese (1.48 ± 0.40 ng/ml) than in normal weight subjects (1.11 ± 0.55 ng/ml) (Fig. 1F; $p < 0.05$).

NPY protein expression in adipose tissue

Immunofluorescent staining revealed that NPY protein, shown in green, was detected in cytoplasm of human adipose tissue (Fig. 2).

Correlations between 2 factors (Table 2)

Age was positively correlated with body weight ($R = 0.522$, $p < 0.01$) and BMI ($R = 0.563$, $p < 0.01$). Body weight and BMI had positive correlations with insulin ($R = 0.639$, $p < 0.001$ and $R = 0.603$, $p < 0.001$, respectively) and HOMA-IR ($R = 0.642$, $p < 0.001$ and $R = 0.617$, $p < 0.001$, respectively), but not glucose (data not shown). Systolic blood pressure, but not diastolic blood pressure, was positively correlated to body weight ($R = 0.397$, $p < 0.05$), BMI ($R = 0.404$, $p < 0.05$), insulin ($R = 0.455$, $p < 0.05$), glucose ($R = 0.434$, $p < 0.05$) and HOMA-IR ($R = 0.509$, $p < 0.01$). NPY gene expression was not significantly correlated with peripheral metabolic factors in both subcutaneous and visceral adipose tissues (data not shown). In addition, the NPY expression in subcutaneous adipose tissue was not correlated to that in the visceral adipose tissue (data not shown). Interestingly, we found a negative correlation between NPY expression in visceral adipose tissue and birth weight of the subjects ($R = -0.875$, $p < 0.05$). A positive correlation was found between Y1R mRNA expression in subcutaneous adipose tissue and serum NPY levels ($R = 0.426$, $p < 0.05$). Y1R gene expression in visceral adipose tissue was positively correlated with body weight ($R = 0.586$, $p < 0.01$), BMI ($R = 0.611$, $p < 0.01$), waist circumference ($R = 0.474$, $p < 0.05$), hip circumference ($R = 0.483$, $p < 0.05$), insulin ($R = 0.539$, $p < 0.01$) and HOMA-IR ($R = 0.480$, $p < 0.05$).

Multivariate regression analysis (Table 3 and 4)

Statistically significant interactions between clinical data and peripheral metabolic factors were found. For some dependent variables, 2-4 models were independently built to avoid collinearity of factors that had highly significant correlations. Taking SBP as the dependent variable, 2 different models were built. For the first model, BMI, plasma insulin, and plasma glucose were used as independent variables which explained 31% ($p < 0.05$) variability of the SBP while body weight was used as the independent variable instead of BMI in the second model which showed 30.7% ($p < 0.05$) variability. DBP had significant interaction with body weight and glucose ($R^2 = 0.215$, $p < 0.05$). Insulin which was set as a dependent variable had significant interactions with body weight and SBP ($R^2 = 0.456$, $p < 0.001$) or BMI and SBP ($R^2 = 0.417$, $p < 0.01$). Glucose had significant interaction only with SBP with R^2 value of 0.188 ($p < 0.05$). By setting HOMA-IR as the dependent variable, 2 models of significant interactions were found by using SBP and body weight ($R^2 = 0.490$, $p < 0.001$) or SBP and BMI ($R^2 = 0.462$, $p < 0.001$) as independent variable. Taking serum NPY as a dependent variable, a significant interaction was observed when setting subcutaneous Y1R and plasma glucose as independent variables ($R^2 = 0.336$, $p < 0.05$). There was no significant interaction between subcutaneous NPY mRNA and measured variables; however, a model was built by setting SBP as independent variables with R^2 value of 0.107 ($p = 0.089$). Visceral NPY mRNA had significant interactions with birth weight and waist circumference ($R^2 = 0.799$, $p < 0.05$) or birth weight and hip circumference ($R^2 = 0.790$, $p < 0.05$) with birth weight being a negative impact factor. Subcutaneous Y1R mRNA showed positive significant interaction with only serum NPY levels ($R^2 = 0.181$, $p < 0.05$). To set visceral Y1R mRNA as a dependent variable, 4 independent models were created. Insulin and BMI or insulin and body

weight set as independent variables showed 45.1 % ($p < 0.01$) and 41.7% ($p < 0.01$) variability of visceral Y1R mRNA, respectively, while HOMA-IR and BMI or HOMA-IR and body weight revealed 42.5% ($p < 0.01$) or 39.6% ($p < 0.01$) variability, respectively.

Discussion

This study focused on comparison of NPY and Y1R mRNA expression in subcutaneous and visceral fat tissues in normal weight and obese humans and its correlation with clinical parameters and peripheral metabolic factors. In this study, 5 subjects in the obese group have fulfilled the metabolic syndrome criteria while no subject in the normal weight group has fallen into the criteria. Obese subjects had higher plasma insulin and HOMA-IR levels than normal weight subjects while plasma glucose was similar between these groups, indicating increased insulin resistance in the obese subjects. Although levels of insulin and HOMA-IR were still in the normal range in obese patients (2-17 µU/ml, 1.8-2.3, respectively), the 2.2 fold (120%) increase in insulin and 2.4 fold (141%) increase in HOMA-IR levels revealed higher risk of insulin resistance in the obese group. Moreover, there were positive correlations between body weight and BMI with insulin, HOMA-IR, but not glucose. In regression analysis, plasma insulin and HOMA-IR could be predicted by setting body weight and SBP or BMI and SBP as independent variables with higher significant levels of interaction detected with body weight. Insulin, glucose and HOMA-IR could be predicted by SBP. SBP was significantly greater in obese than in normal weight subjects by 1.2 fold and mean SBP in the obese group was 133.54 mmHg which is higher than 130 mmHg (1 diagnostic criteria of metabolic syndrome criteria) [57]. Moreover, SBP positively correlated with body weight and BMI which is suggestive of increased hypertension in obese humans

[58]. Mean DBP was also significantly greater in obese than normal weight subjects; however, the levels of mean DBP in obese subjects were still in the normal range. In regression analysis, SBP had significant interactions with BMI, insulin, and glucose or body weight, insulin, and glucose while DBP had significant interaction with only body weight and glucose. As a result, DBP might be a less sensitive indicator for detection of metabolic syndrome than SBP.

NPY mRNA expression was detected in human adipose tissues. This study also showed NPY protein in adipose tissue by immunofluorescent staining confirming the presence of NPY protein in the cytoplasm of adipocytes. However, comparisons between obese and normal weight subjects or between subcutaneous and visceral adipose tissue have not been done due to the insufficiency of the tissue. NPY mRNA was significantly higher in obese group than in normal weight group with increased levels of expression in obese people of 2.7 fold in subcutaneous and by 4 fold in visceral adipose tissues indicating that high NPY expression especially in visceral adipose tissues was found in obese subjects who had higher amount of body fat. The results might be explained by evidences from previous studies that NPY increases preadipocyte proliferation, differentiation, and adipogenesis (lipid-filling) via Y2 receptor activation in under chronic stress in vitro [59]. Furthermore, NPY is involved in fat proliferation via Y1 and Y2 receptors [44, 45] and fat differentiation via Y2 receptor [44] in rodents, causing increased amount of total body fat. In addition, human adipocytes treated with recombinant (rh) NPY reduced glycerol release, which is an index of lipolysis [42], thus confirming an antilipolytic action of NPY. Chronic stress also induces NPY release [44], increased NPY mRNA [60], and up-regulated Y2R expression in abdominal fat in high-fat, high-sugar fed mice [44], resulting in metabolic syndrome-like symptoms of abdominal obesity, fat inflammation, hepatic steatosis [44], hyperlipidemia [61], and hypertension [60].

Interestingly, fat accumulation and metabolic complications can be prevented or reversed by pharmacological Y2R inhibition at local intra-fat or adenoviral Y2R knock-down [44]. As a result, an increase in NPY mRNA expression in obese humans might be related to increased obesity in these subjects. The result of this study was different from a previous study in German subjects showing that NPY gene expression in both visceral and subcutaneous adipose tissues was not significantly different when compared between patients who underwent laparoscopic gastric banding surgery (obese group) and patients who underwent laparoscopic fundoplication surgery (control groups) [43]; however, the BMI of the subjects in the control group was not indicated as lower than 30 kg/m^2 and the mean BMI ± SD of this group was 27 ± 3.5 kg/m^2. As a result, the subjects in the previous study [43] that had BMI of more than 30 kg/m^2 might have been recruited in the control group leading to a different finding from the result of this study. Furthermore, the concentration of serum NPY also showed significant higher levels in obese when compared with normal weight subjects. Our result was different from the previous study in German subjects undergoing laparoscopic fundoplication surgery [43] as discussed earlier. It is important to note that NPY is not released only from adipose tissue but also from other tissues such as adrenal medulla [35], sympathetic ganglia and tissues receiving dense sympathetic innervations [36-40]. In addition, NPY levels were 3 fold higher in cerebrospinal fluid (CSF) when compared with the blood stream and there was no correlation between CSF and plasma NPY levels [62]. NPY mRNA and serum NPY levels were not correlated with any clinical parameters (data not shown) suggesting that NPY might not be directly or proportionally related to those parameters. In regression analysis, there was no significant interaction between NPY mRNA and other measured data. Our results were similar to a previous study of German undergoing laparoscopic surgery [43] showing that plasma NPY levels were not correlated with SBP, DBP nor BMI. Previous studies showed that recombinant human (rh) insulin treatment in

human abdominal subcutaneous adipocytes increased NPY secretion across the concentration range [42], but not in a dose-dependent manner. Furthermore insulin injection increased NPY mRNA expression in visceral adipose tissue of lean Zucker rats but not in obese Zucker rats [45]. Consequently, insulin appears to regulate NPY in subcutaneous adipocytes and visceral adipose tissue but not in a direct proportion nor in a wide variety of different cohort analyses. In our study, although NPY mRNA expression in visceral adipose tissue was higher in obese than normal weight subjects, a lack of statistically significant correlation of these 2 factors was observed. These results might be explained in that insulin might not be proportionally related to NPY mRNA expression in adipose tissue of obese and normal weight subjects. As NPY acts via its receptors, the action of NPY on target cells might be better explained by receptor expression.

NPY mRNA expression was higher in visceral than in subcutaneous adipose tissues in obese (3.8 fold), normal weight (2.5 fold) and overall subjects (3.8 fold) implying that visceral adipose tissue might synthesize NPY more than subcutaneous adipose tissue. Although it has been revealed that NPY protein was expressed in subcutaneous adipose tissue almost 2 fold higher than in omental adipose tissue in Caucasian female subjects [42], our study couldn't present the comparison of protein expression levels because of the scarcity of the sample. This different finding might be caused by posttranslational process or endocytosis of NPY from circulation into fat cells or difference in ethnicity and race. Furthermore, in the previous study [42], comparisons of protein expression were made between subcutaneous and omental adipose tissues obtained from 8 subjects, while in our study the tissues were obtained from 26 subjects. Basically, there are differences between visceral and subcutaneous fat depots, not only in regard to anatomical location but also in terms of gene expressions and functional differences. Previous studies showed that visceral adipose tissue was more capable of

synthesis and of releasing adipokines, such as TNF-α [63], IL-6 [63, 64], adiponectin [65] and angiotensinogen [66], when compared to subcutaneous fat. Functional differences of visceral and subcutaneous fat depots demonstrated that visceral adipocytes were highly elevated in lipolytic activity [67] causing both an elevation of liver uptake free fatty acids [68] and gluconeogenesis [69, 70]. This leads to a reduction of hepatic insulin extraction by inhibiting insulin breakdown [71] resulting in peripheral hyperinsulinemia [72-74]. Obviously, increased NPY expression in visceral adipose tissue might be closely related with insulin resistance [75]. In this study, there was a strong negative correlation between NPY mRNA expression in visceral fat and birth weight. In addition, visceral NPY mRNA had highly significant interactions with birth weight and waist circumference or birth weight and BMI with 79% variability of prediction suggesting that birth weight might be a negative indicator for determining visceral NPY mRNA in adulthood. However, please note that total number of subjects was only 10 as only 10 subjects had their recoded birth weight data. It has been reported that intrauterine growth restriction (IUGR), resulting in low birth weight and rapid weight gain during infancy [76-78] caused visceral adiposity in rats [79-81], obesity in children [82, 83], increased risk of metabolic disorders [76, 77, 84] as well as up-regulated NPY mRNA expression in hypothalamus of IUGR sheep (Anukulkitch C., unpublished data) and rats [85, 86]. Furthermore, previous studies have demonstrated IUGR had strong relationships with later development of metabolic syndrome in middle and late life in humans, including increased and more centralized distribution of fat mass [87-89] and many other obesity-related diseases with unrelated to age, current weight, BMI, gender, ethnic origin, smoking, family history of diabetes and oral contraception and level of exercise at the time of the study [87-94]. From these multiple evidences, there is a hypothesis drawn that adult disease might arise in utero, in part as a result of changes and reprogramming of the hypothalamic–pituitary–adrenal axis, insulin-signaling pathways, abnormalities in the

circulating concentrations of insulin, catecholamines, cortisol, GH, and insulin-like growth factors (IGFs) [95-97] and, in addition, changes in hormone bioavailability in adulthood [96]. Thus, negative correlations between birth weight and NPY mRNA expression in visceral fat in this study might indicate that low birth weight is related to increased central obesity and metabolic syndrome [87-94]. As a result birth weight might be an indicator for prediction of visceral NPY mRNA expression in adulthood.

Comparison between obese and normal weight subjects showed that Y1R mRNA expression was 4 fold significantly greater in obese than in normal weight subjects in visceral adipose tissue but not in subcutaneous adipose tissue. Moreover, there were positive correlations between Y1R mRNA expression in visceral adipose tissue and body weight, BMI, waist and hip circumference. These results indicated that high expression of Y1R in visceral adipose tissue might be closely associated with obesity. Previous studies showed that injection of Y1R antagonist reduced body weight [51] as well as Y1R deletion and caused a body weight reduction in male and female mice [98]. It has been revealed that intracerebroventricular (icv) injected NPY significantly reduced food intake in Y1 receptor-deficient mice [99] as well as that administration of Y1R suppressed food intake in high-fat diet-fed obese mice [51]. Furthermore, NPY stimulated rat preadipocyte proliferation via Y1R mediated through the activation of ERK1/2 [45], and the strong antilypolytic effect of NPY was blocked by specific Y1R antagonists [100]. As a result, Y1R expression, especially in visceral adipose tissue, might be closely related to increased obesity and fat accumulation. Our data showing comparable levels of expression of Y1R mRNA in subcutaneous tissue between obese and normal weight subjects were not similar to another publication reporting a significantly higher expression of Y1R in subcutaneous adipose tissue in obese humans when compared to the lean cohort [49]. This dissimilar result might be explained by the use of different groups

for comparison. In our study, we compared obese and normal weight subjects while another study [49] compared obese and lean individuals. Moreover, differences in BMI cut-off points and ethnicity might also be associated reasons. Y1R expression in visceral fat was also positively correlated with insulin levels and HOMA-IR, indicating associations between NPY action via Y1R with hyperinsulinemia and insulin resistance. Regression analysis showed that Y1R mRNA in visceral fat had significant interactions with BMI or bodyweight and insulin or HOMA-IR, suggesting that increased obesity and insulin/insulin resistance are factors involved in increased visceral Y1R mRNA. It has been reported that a single dose icv administration of Y1R agonist increased plasma insulin levels at 15 minutes, 1 hour and 2 hours after injection in satiated Long-Evan rats with and without the presence of food [101] indicating that Y1R is closely related to hyperinsulinemia. A previous study reported that NPY and Y1R agonist reduced insulin induced translocation of GLUT4 from intracellular stores to cell surface in 3T3-L1 adipocytes and that Y1R antagonist prevents the reduction, indicating that NPY increased insulin resistance via Y1R [102]. In conclusion, Y1R mRNA expression in visceral fat is closely related to obesity and metabolic syndrome.

Y1R mRNA expression was highly expressed in subcutaneous adipose tissue when compared to visceral adipose tissue in overall, obese and normal weight subjects, possibly reflecting that Y1R was more highly synthesized in subcutaneous than visceral adipose tissue. Unfortunately, we didn't have enough fat tissue to confirm a protein expression study. It has been reported that Y1R protein expression indicated no significant difference between subcutaneous and omental human adipose tissues [49], but the number of subjects in this study was only 6. Additionally, microarray study showed highest expression of Y1R to be in subcutaneous adipose tissue at least 5 fold more than in other tissues (including brain, adrenal gland and uterus) indicating that NPY mediated its action via Y1R mainly at subcutaneous

adipose tissue [49]; however, the expression in visceral adipose tissue was not mentioned in that study [49]. Y1R mRNA expression in subcutaneous adipose tissue positively correlated with serum NPY. Regression analysis also showed that serum NPY had significant interactions with Y1R mRNA in subcutaneous fat and glucose. Moreover, serum NPY, NPY mRNA, Y1R mRNA in subcutaneous fat tissue did not show any statistically significant correlation among body weight, BMI and clinical parameters. These results can be interpreted that NPY levels in the circulation might be increased along with increased NPY action via Y1R in subcutaneous adipose tissue and increased plasma glucose but not obesity or other clinical parameters.

In conclusion, this study revealed that obese people have higher SBP, DBP, insulin levels, insulin resistance, NPY mRNA expression in both subcutaneous and visceral adipose tissue, and, additionally, Y1R mRNA expression in visceral adipose tissue. Up-regulation of Y1R especially in visceral adipose tissue had positive correlations with, body weight, waist and hip circumferences, insulin and HOMA-IR, so Y1R expression in visceral adipose tissue might be an indicator of increased risk of metabolic syndrome. Further investigation about the blocking Y1 receptor subtype, especially on adipose tissue, may reveal strategies for risk reduction in metabolic syndrome and prevention or treatment of obesity.

Acknowledgments

This study is supported by Faculty of Medicine Siriraj Hospital Research Fund, Siriraj Graduate Thesis Scholarship, and Grant for New Scholar (co-funded by the Thailand Research Fund (TRF)). We thank Weerapat Jumpol for providing chart work and data

analysis. We also offer our sincere gratitude to Smarn Onreabroi, Namoiy Semprasert, and the staff and nurses from the Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University for blood and tissue collection.

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Table and Figure legends

Table 1 Distribution of patient's SBP, DBP, plasma levels of glucose, insulin, and HOMA-IR for obese and normal weight subjects**,** Values are expressed as mean ± SD., *$p < 0.05$, **$p < 0.01$ compared with normal weight

Table 2 Correlation analyses between 2 factors, n = 30 except $^{\#}$ n =10, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 3 Multivariate regression analysis of clinical parameters and peripheral metabolic factors, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 4 Multivariate regression analysis of serum NPY, NPY and Y1R mRNA in subcutaneous and visceral adipose tissue, $^{\#}$ calculated from n =10, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Figure 1 Mean (± SD) NPY and Y1R mRNA expression in subcutaneous and visceral adipose tissue specimens and serum NPY. Panel A shows gel electrophoresis revealing expression of LRP-10 (amplicon size 169 bp), NPY (amplicon size 102 bp), and Y1R (amplicon size 222 bp) from fat tissues. Panel B presents NPY mRNA expression compared between obese and normal weight subjects in subcutaneous and visceral fat pads. Panel C presents NPY mRNA expression compared between subcutaneous and visceral fat pads in obese, normal weight, and overall subjects. Panel D presents Y1R mRNA expression compared between obese and normal weight subjects in subcutaneous and visceral fat pads. Panel E presents Y1R mRNA expression compared between subcutaneous and visceral fat pads in obese, normal weight, and overall subjects. Panel F presents serum NPY

concentrations compared between obese and normal weight groups, $*p < 0.05$, $**p < 0.01$ compared between groups.

Figure 2 Immunofluorescent staining. Panel A shows staining of NPY in adipose tissue. Panel B shows negative control with omission of primary antibody. Green colors represent staining of secondary antibody and blue color represents staining of nuclei.

Figure

Table 1

Clinical parameters	Obese	Normal weight
number of the subjects that fulfilled the metabolic syndrome criteria	5	0
Systolic blood pressure, mmHg (mean ± SD)	133.54 ± 14.26**	112.88 ± 6.08
Diastolic blood pressure, mmHg (mean ± SD)	74.87 ± 8.33*	66.67 ± 5.20
Plasma glucose, mg/dl (mean ± SD)	83.53 ± 7.74	84.38 ± 8.65
Plasma insulin, μU/ml (mean ± SD)	8.13 ± 5.61*	3.69 ± 1.75
HOMA-IR (mean ± SD)	1.64 ± 1.14*	0.68 ± 0.36

Table 2

Factors	Pearson correlation (R)	R^2	p value
Age-body weight	0.522**	0.272	< 0.01
Age-BMI	0.563**	0.317	< 0.01
Body weight-SBP	0.397*	0.158	< 0.05
Body weight-insulin	0.639**	0.408	< 0.001
Body weight-HOMA-IR	0.642**	0.412	< 0.001
BMI-SBP	0.404*	0.163	< 0.05
BMI-insulin	0.603**	0.364	< 0.001
BMI-HOMA-IR	0.617**	0.381	< 0.001
SBP-insulin	0.455*	0.207	< 0.05
SBP-glucose	0.434*	0.188	< 0.05
SBP-HOMA-IR	0.509**	0.259	< 0.01
Visceral NPY mRNA-birth weight[#]	-0.875**	0.766	< 0.05
Subcutaneous Y1R mRNA-serum NPY	0.426*	0.181	< 0.05
Visceral Y1R mRNA-Body weight	0.586**	0.343	< 0.01
Visceral Y1R mRNA-BMI	0.611**	0.373	< 0.01
Visceral Y1R mRNA-waist circumference	0.474*	0.225	< 0.05
Visceral Y1R mRNA-hip circumference	0.483*	0.233	< 0.05
Visceral Y1R mRNA-Insulin	0.539**	0.291	< 0.01
Visceral Y1R mRNA-HOMA-IR	0.480*	0.230	< 0.05

[#]n=10

Table 3

Dependent variable	Model				coefficient	standard error	T-value	p value
	R	R2	p value					
SBP1	.556	.310	.024*	(Constant)	80.735	18.245	4.425	.000***
				BMI	.505	.568	.888	.383
				insulin	.801	.751	1.067	.296
				glucose	.284	.171	1.662	.109
SBP2	.554	.307	.025*	(Constant)	82.155	17.667	4.650	.000***
				body weight	.175	.209	.836	.411
				insulin	.776	.784	.990	.332
				glucose	.292	.171	1.710	.100
DBP	.463	.215	.043*	(Constant)	52.166	8.625	6.049	.000***
				body weight	.164	.089	1.836	.078
				glucose	.127	.088	1.442	.161
insulin1	.675	.456	.000***	(Constant)	-11.643	5.282	-2.204	.037*
				body weight	.152	.044	3.451	.002**
				SBP	.069	.046	1.516	.142
insulin2	.646	.417	.001**	(Constant)	-12.710	5.528	-2.299	.030*
				BMI	.394	.129	3.059	.005**
				SBP	.073	.047	1.544	.135
glucose	.434	.188	.019*	(Constant)	31.769	22.884	1.388	.176
				SBP	.456	.182	2.504	.019*
HOMA-IR1	.700	.490	.000***	(Constant)	-3.829	1.360	-2.815	.009**
				SBP	.023	.012	1.971	.059
				Body weight	.039	.011	3.431	.002**
HOMA-IR2	.679	.462	.000***	(Constant)	-4.119	1.412	-2.917	.007**
				SBP	.024	.012	1.971	.059
				BMI	.103	.033	3.130	.004**

Table 4

Dependent variable	Model				coefficient	standard error	T-value	p value
	R	R^2	p value					
serum NPY	0.58	0.336	.017*	(Constant)	-0.007038	0.531	-0.013	0.99
				subcutaneous Y1R	0.185087	0.074	2.511	.021*
				glucose	0.012266	0.006	2.158	.043*
subcutaneous NPY mRNA	0.328	0.107	0.089	(Constant)	0.001503	0.001	2.55	.017*
				SBP	-0.000008	0	-1.768	0.089
visceral NPY mRNA1	0.894	0.799	.041*	(Constant)	0.246884	0.153	1.611	0.182
				birth weight	-0.000128	0	-3.934	.017*
				waist circumference	0.001266	0.002	0.813	0.462
visceral NPY mRNA2	0.889	0.79	.044*	(Constant)	0.162327	0.288	0.563	0.604
				birth weight	-0.000118	0	-3.325	.029*
				hip circumference	0.001695	0.002	0.685	0.531
subcutaneous Y1R mRNA	0.426	0.181	.043*	(Constant)	-0.067974	0.708	-0.096	0.924
				serum NPY	1.056378	0.49	2.156	.043*
visceral Y1R mRNA1	0.671	0.451	.002**	(Constant)	-0.932816	0.422	-2.21	0.038
				insulin	0.043671	0.025	1.713	0.101
				BMI	0.044521	0.018	2.47	.022*
visceral Y1R mRNA2	0.646	0.417	.003**	(Constant)	-0.705212	0.384	-1.838	0.08
				insulin	0.043937	0.027	1.626	0.119
				body weight	0.014517	0.007	2.128	.045*
visceral Y1R mRNA3	0.652	0.425	.003**	(Constant)	-0.9621	0.431	-2.233	0.037
				HOMA-IR	0.145108	0.106	1.373	0.184
				BMI	0.048279	0.018	2.671	.014*
visceral Y1R mRNA4	0.629	0.396	.005**	(Constant)	-0.739743	0.388	-1.905	0.071
				HOMA-IR	0.148606	0.11	1.356	0.19
				body weight	0.016080	0.007	2.402	0.026

Fig. 1

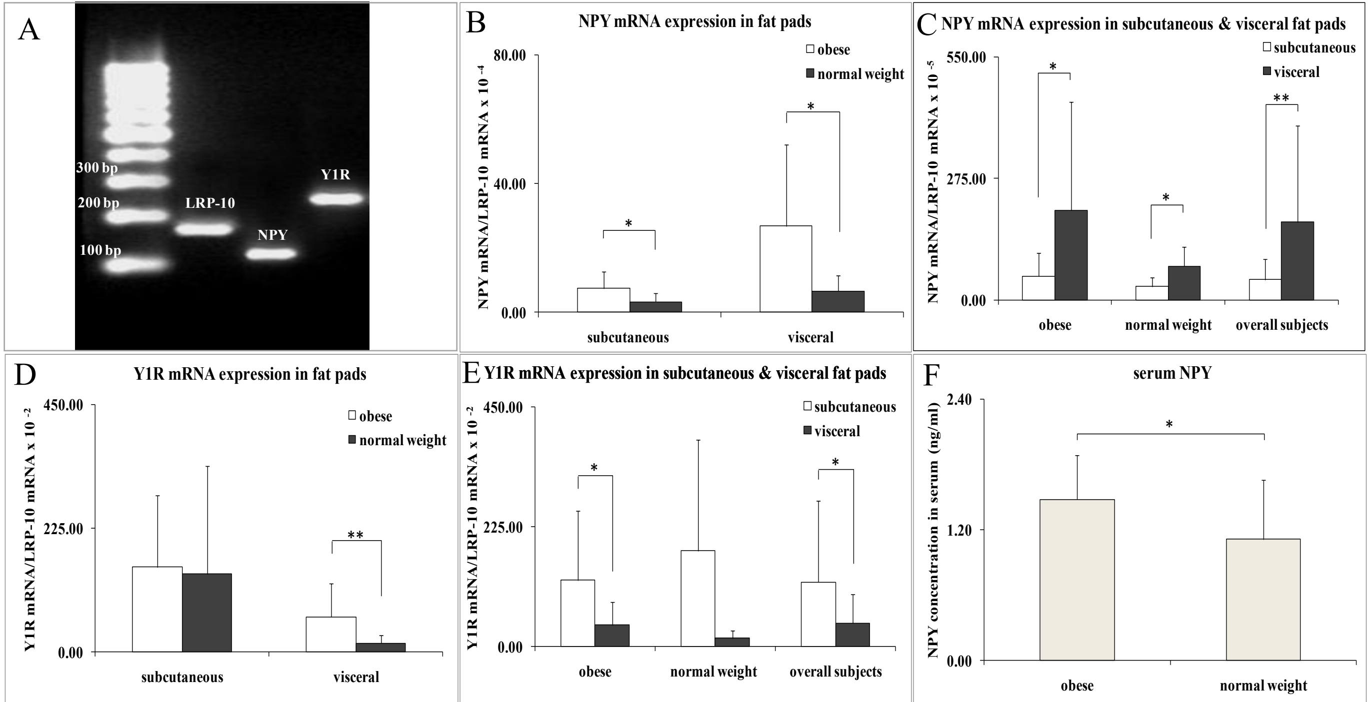
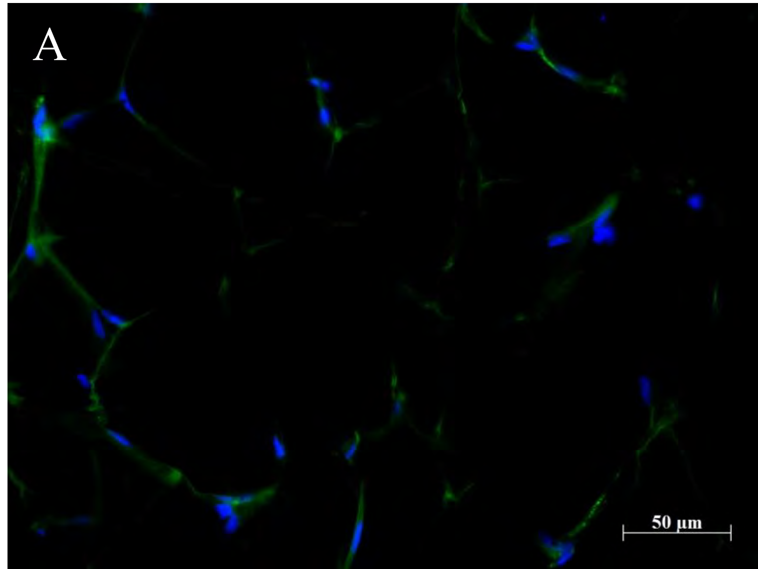
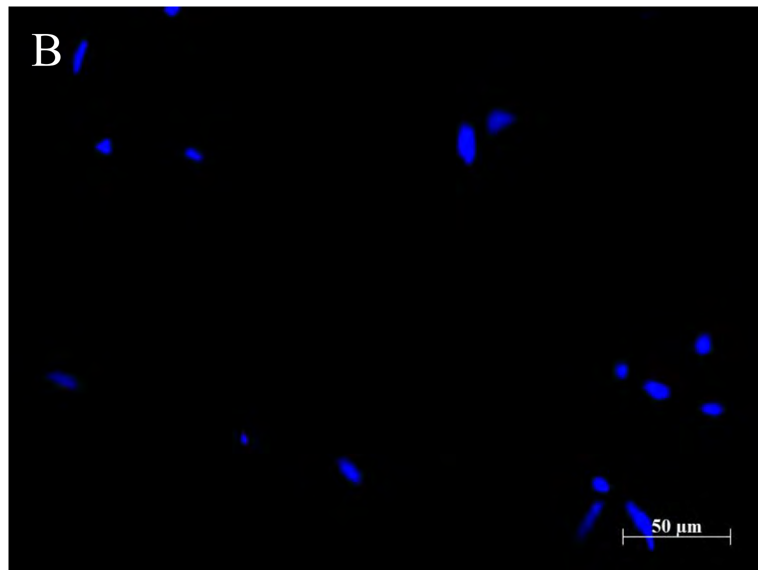


Fig.2





Response to Reviewers:

Reviewer #1: I am grateful for the author's kind reply to all comments and for the data they have added. As for the sample size in the correlation analysis between expression levels of NPY and birth weight, n=10, I would suggest just to mention it in the text (for instance, in Table 2 legend) since the statistical power seems good enough.

Response: We are very grateful to the reviewer's helpful suggestion. We have now added $^{\#}$ in Table 2 and in figure legend to indicate n = 10 and have added the information in the discussion section as follows: "However please be aware that total number of subjects was only 10 as only 10 subjects had their birth weight data." (page 16)

There are still some more comments:
1. The 2-<DELTA>Ct method allows comparing expression levels of different genes only when similar PCR efficiency is assumed. The authors should show how they checked the amplification efficiency. On the other hand, if there is no comparison among genes along the manuscript, it would be more accurate to use the 2-<DELTA><DELTA>Ct.

Response: We thank the reviewer for raising this point. Actually, we tested the efficiency of each primer by amplification of real-time PCR products in 5 dilutions of cDNA and the results of the real-time PCR reaction are shown as amplification plots in Figure 1. The hallmarks of a real-time PCR assay optimization include 1) linear standard curve ($R^2 > 0.98$), 2) high amplification efficiency (90-105%), and 3) consistency across replicate reactions (1). For our study, an example of the standard curve with the C_T plotted against the log of the starting quantity of template of each dilution is shown in Figure 2. The R^2 value of linear standard curve was 0.9969. The slope was calculated from regression line and the efficiency (E) was calculated according to the equation; $E = 10^{-1/slope}$. A percentage of efficiency (%E) is the percentage of template that was amplified in each cycle calculated by the following equation; %E = (E-1) x 100%. Efficiency close to 100% is the best indicator of robust reproducible assay and the amplification efficiency of 90-105% is accepted (1). Our study showed %E at 91.80213 which was accepted as a robust, reproducible assay as shown in the calculations below.

$$
\begin{aligned}
E &= 10^{-1/slope} = 10^{-1/3.5354} \\
E &= 1.9180213 \\
\%E &= (E-1) \times 100\% \\
&= (1.9180213-1) \times 100\% \\
&= 91.80213
\end{aligned}
$$

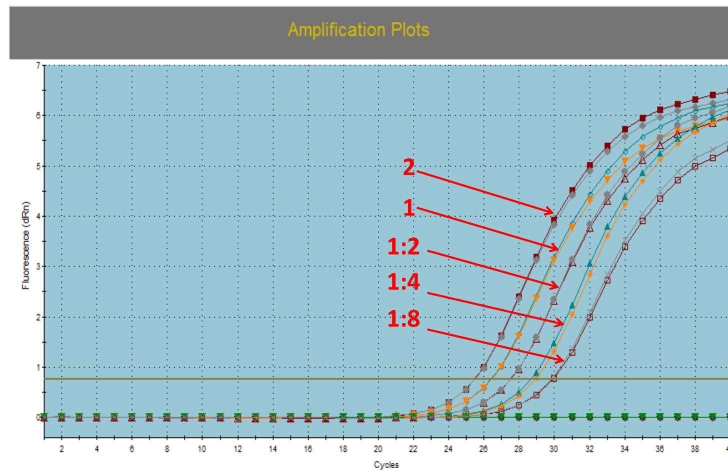


Figure 1 the amplification plots showing amplification products of 5 dilutions of DNA from human adipose tissue

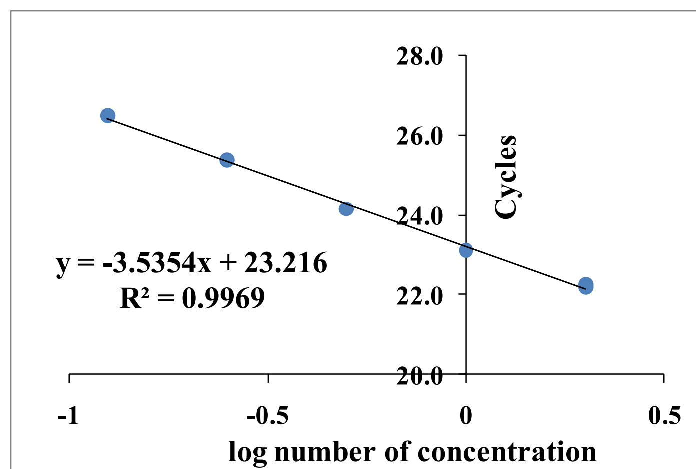


Figure 2 the standard curve showing C_T plotted against the log of the starting quantity of template of each dilution

Furthermore, we would like to find correlations among gene expression, clinical data, and peripheral metabolic factors, so the results obtained from the $2^{-\Delta CT}$ method would be beneficial in this aspect.

Now, we have added this information in the method section in our manuscript as follows:

The hallmarks of a real-time PCR assay optimization include 1) linear standard curve ($R^2 > 0.98$), 2) high amplification efficiency (90-105%), and 3) consistency across replicate reactions (1). The efficiency of each primer was tested by amplification of real-time PCR products in 5 dilutions of cDNA. For our study, the R^2 value of linear standard curve was 0.9969 and the percentage of efficiency was 91.80213 which was consistent across duplicate reactions. Thus, our real-time PCR assay was accepted as a robust, reproducible assay. As a result, the $2^{-\Delta CT}$ method was applied as a comparative method of quantification. (page 6)

2. I couldn't find the reference for the sentence "Chronic stress also induces NPY release, hepatic steatosis and hypertension." (minor comment 2 in the original version; now page 14, lines 5-8).

Response: Thank you for the reviewer's comment. We have now added the new references in our manuscript as follows:
New statement: Chronic stress also induces NPY release (2), increased NPY mRNA (3), and up-regulated Y2R expression in abdominal fat in high-fat, high-sugar fed mice (2), resulting in metabolic syndrome-like symptoms of abdominal obesity, fat inflammation, hepatic steatosis (2), hyperlipidemia (4), and hypertension (3).

3. The discussion is still too long and I found it really difficult to go through. The authors still need to focus in the main issue, NPY, not only adding information (as they have already done), but also reducing other issues more related to obesity than to NPY. I would remove the discussion on metabolic parameters of obese subjects (first paragraph) and only mention a brief description.

Response: We thank the reviewer for recommendation. Now, we have changed the first paragraph of discussion section as reviewer's suggestion.
New statement:

This study focused on comparison of NPY and Y1R mRNA expression in subcutaneous and visceral fat tissues in normal weight and obese humans and its correlation with clinical parameters and peripheral metabolic factors. In this study, 5 subjects in the obese group have fulfilled the metabolic syndrome criteria while no subject in the normal weight group has fallen into the criteria. Obese subjects had higher plasma insulin and HOMA-IR levels than normal weight subjects while plasma glucose was similar between these groups, indicating increased insulin resistance in the obese subjects. Although levels of insulin and HOMA-IR were still in the normal range in obese patients (2-17 μU/ml, 1.8-2.3, respectively), the 2.2 fold (120%) increase in insulin and 2.4 fold (141%) increase in HOMA-IR levels revealed higher risk of insulin resistance in the obese group. Moreover, there were positive correlations between body weight and BMI with insulin, HOMA-IR, but not glucose. In regression analysis, plasma insulin and HOMA-IR could be predicted by setting body weight and SBP or BMI and SBP as independent variables with higher significant levels of interaction detected with body weight. Insulin, glucose and HOMA-IR could be predicted by SBP. SBP was significantly greater in obese than in normal weight subjects by 1.2 fold and mean SBP in the obese group was 133.54 mmHg which is higher than 130 mmHg (1 diagnostic criteria of metabolic syndrome criteria) (5). Moreover, SBP positively correlated with body weight and BMI which is suggestive of increased hypertension in obese humans (6). Mean DBP was also significantly greater in obese than normal weight subjects; however, the levels of mean DBP in obese subjects were still in the normal range. In regression analysis, SBP had high significant interactions with BMI, insulin, and glucose or body weight, insulin, and glucose while DBP had low significant interaction with only body weight and glucose. As a result, DBP might be a less sensitive indicator for detection of metabolic syndrome than SBP.

4. Correlations between two clinical/anthropometric variables are informative but regression analysis should be performed to see if a variable actually affects the other. In my opinion, this is not the purpose of this study. By contrast, a description in Table 1 of the subjects that fulfill the Metabolic Syndrome criteria should better focus the reader on NPY.

Response: Thank you the reviewer for valuable comments. Now, we have added the number of subjects that fulfilled the metabolic syndrome criteria in table 1 as follows:

Table 1 Distribution of patient's SBP, DBP, plasma levels of glucose, insulin, and HOMA-IR for obese and normal weight subjects

Clinical parameters	Obese	Normal weight
number of the subjects that fulfilled the metabolic syndrome criteria	5	0
Systolic blood pressure, mmHg (mean ± SD)	133.54 ± 14.26**	112.88 ± 6.08
Diastolic blood pressure, mmHg (mean ± SD)	74.87 ± 8.33*	66.67 ± 5.20
Plasma glucose, mg/dl (mean ± SD)	83.53 ± 7.74	84.38 ± 8.65
Plasma insulin, µU/ml (mean ± SD)	8.13 ± 5.61*	3.69 ± 1.75
HOMA-IR (mean ± SD)	1.64 ± 1.14*	0.68 ± 0.36

Values are expressed as mean ± SD.
**$p < 0.01$, *$p < 0.05$ compared with normal weight

We have now added a statement in discussion section at page 12 as follows:
"In this study, 5 subjects in the obese group have fulfilled the metabolic syndrome criteria while no subject in the normal weight group has fallen into the criteria."

The regression analysis models have now been analyzed as shown in table 3. We have also added the regression analysis for HOMA-IR, insulin, SBP, DBP, and glucose in the result section (page 11) as follows:

New statement:

Multivariate regression analysis (Table 3 and 4)

Statistically significant interactions between clinical data and peripheral metabolic factors were found. For some dependent variables, 2-4 models were independently built to avoid collinearity of factors that had highly significant correlations. Taking SBP as the dependent variable, 2 different models were built. For the first model, BMI, plasma insulin, and plasma glucose were used as independent variables which explained 31% ($p < 0.05$) variability of the SBP while body weight was used as the independent variable instead of BMI in the second model which showed 30.7% ($p < 0.05$) variability. DBP had significant interaction with body weight and glucose ($R^2 = 0.215$, $p < 0.05$). Insulin, which was set as a dependent variable, had significant interactions with body weight and SBP ($R^2 = 0.456$, $p < 0.001$) or BMI and SBP ($R^2 = 0.417$, $p < 0.01$). Glucose had significant interaction only with SBP with R^2 value of 0.188 ($p < 0.05$). By setting HOMA-IR as the dependent variable, 2 models of significant interactions were found by using SBP and body weight ($R^2 = 0.490$, $p < 0.001$) or SBP and BMI ($R^2 = 0.462$, $p < 0.001$) as independent variable.

The new information has also been added in the discussion section as in the highlighted areas (page 12-19).

Table 3 Multivariate regression analysis of clinical parameters and peripheral metabolic factors, *p < 0.05, **p < 0.01, ***p < 0.001

Dependent variable	Model				coefficient	standard error	T-value	p value
	R	R2	p value					
SBP1	.556	.310	.024*	(Constant)	80.735	18.245	4.425	.000***
				BMI	.505	.568	.888	.383
				insulin	.801	.751	1.067	.296
				glucose	.284	.171	1.662	.109
SBP2	.554	.307	.025*	(Constant)	82.155	17.667	4.650	.000***
				body weight	.175	.209	.836	.411
				insulin	.776	.784	.990	.332
				glucose	.292	.171	1.710	.100
DBP	.463	.215	.043*	(Constant)	52.166	8.625	6.049	.000***
				body weight	.164	.089	1.836	.078
				glucose	.127	.088	1.442	.161
insulin1	.675	.456	.000***	(Constant)	-11.643	5.282	-2.204	.037*
				body weight	.152	.044	3.451	.002**
				SBP	.069	.046	1.516	.142
insulin2	.646	.417	.001**	(Constant)	-12.710	5.528	-2.299	.030*
				BMI	.394	.129	3.059	.005**
				SBP	.073	.047	1.544	.135
glucose	.434	.188	.019*	(Constant)	31.769	22.884	1.388	.176
				SBP	.456	.182	2.504	.019*
HOMA-IR1	.700	.490	.000***	(Constant)	-3.829	1.360	-2.815	.009**
				SBP	.023	.012	1.971	.059
				Body weight	.039	.011	3.431	.002**
HOMA-IR2	.679	.462	.000***	(Constant)	-4.119	1.412	-2.917	.007**
				SBP	.024	.012	1.971	.059
				BMI	.103	.033	3.130	.004**

5. as for the correlation analysis for serum NPY, NPY MRNA and Y1R, a regression analysis or general lineal model would be more informative.

Response: We would like to thank the reviewer for this helpful recommendation. We have now added the regression analysis for serum NPY, NPY mRNA, and Y1R mRNA in table 4 and in the multivariate regression analysis of result section.

Taking serum NPY as a dependent variable, a significant interaction was observed when setting subcutaneous Y1R and plasma glucose as independent variables ($R^2 = 0.336$, $p < 0.05$). There was no significant interaction between subcutaneous NPY mRNA and measured variables; however, a model was built by setting SBP as independent variables with R^2 value of 0.107 ($p = 0.089$). Visceral NPY mRNA had significant interactions with birth weight and waist circumference ($R^2 = 0.799$, $p < 0.05$) or birth weight and hip circumference ($R^2 = 0.790$, $p < 0.05$) with birth weight being a negative impact factor. Subcutaneous Y1R mRNA showed positive significant interaction with only serum NPY levels ($R^2 = 0.181$, $p < 0.05$). To set visceral Y1R mRNA as a dependent variable, 4 independent models were created. Insulin and BMI or insulin and body weight set as independent variables showed 45.1 % ($p < 0.01$) and 41.7% ($p < 0.01$) variability of visceral Y1R mRNA, respectively while HOMA-IR and BMI or HOMA-IR and body weight revealed 42.5% ($p < 0.01$) or 39.6% ($p < 0.01$) variability, respectively.

The new information has also been added in the discussion section as the highlighted areas (page 12-19).

Table 4 Multivariate regression analysis of serum NPY, NPY and Y1R mRNA in subcutaneous and visceral adipose tissue, # calculated from n =10, *p < 0.05, **p < 0.01, ***p < 0.001

Dependent variable	Model				coefficient	standard error	T-value	p value
	R	R^2	p value					
serum NPY	0.58	0.336	.017*	(Constant)	-0.007038	0.531	-0.013	0.99
				subcutaneous Y1R	0.185087	0.074	2.511	.021*
				glucose	0.012266	0.006	2.158	.043*
subcutaneous NPY mRNA	0.328	0.107	0.089	(Constant)	0.001503	0.001	2.55	.017*
				SBP	-0.000008	0	-1.768	0.089
visceral NPY mRNA1	0.894	0.799	.041*	(Constant)	0.246884	0.153	1.611	0.182
				birth weight	-0.000128	0	-3.934	.017*
				waist circumference	0.001266	0.002	0.813	0.462
visceral NPY mRNA2	0.889	0.79	.044*	(Constant)	0.162327	0.288	0.563	0.604
				birth weight	-0.000118	0	-3.325	.029*
				hip circumference	0.001695	0.002	0.685	0.531
subcutaneous Y1R mRNA	0.426	0.181	.043*	(Constant)	-0.067974	0.708	-0.096	0.924
				serum NPY	1.056378	0.49	2.156	.043*
visceral Y1R mRNA1	0.671	0.451	.002**	(Constant)	-0.932816	0.422	-2.21	0.038
				insulin	0.043671	0.025	1.713	0.101
				BMI	0.044521	0.018	2.47	.022*
visceral Y1R mRNA2	0.646	0.417	.003**	(Constant)	-0.705212	0.384	-1.838	0.08
				insulin	0.043937	0.027	1.626	0.119
				body weight	0.014517	0.007	2.128	.045*
visceral Y1R mRNA3	0.652	0.425	.003**	(Constant)	-0.9621	0.431	-2.233	0.037
				HOMA-IR	0.145108	0.106	1.373	0.184
				BMI	0.048279	0.018	2.671	.014*
visceral Y1R mRNA4	0.629	0.396	.005**	(Constant)	-0.739743	0.388	-1.905	0.071
				HOMA-IR	0.148606	0.11	1.356	0.19
				body weight	0.016080	0.007	2.402	0.026

6. Which is the rationale for selecting the receptor Y1R? Did the authors also analyse Y2R mRNA levels?

Response: We thank the reviewer for this comment. We selected the Y1R because previous study highlighted that Y1R involved in fat proliferation of primary cultures of rat preadipocytes as well as murine 3T3-L1 preadipocytes cell (7) and the strong antilipolytic effect of NPY was blocked by specific Y1R antagonists (8). As a result, Y1R might be differently expressed between obese and normal weight subjects. Actually, we did the Y2R, Y5R, and PYY genes expressions in adipose tissues. However, the data could not be presented in this manuscript as the results were submitted to other publication.

Reviewer #5: The authors responded well to the previous review. Data has been added to expand the depth of the study. The manuscript has been edited to restrict the over-interpretation of the data.

Response: We would like to offer our gratefulness and deep appreciation for the reviewer's response.

References

1. Bio-Rad Laboratories I. key concepts of real-time PCR. 2006:3-5.

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7. Yang K, Guan H, Arany E, Hill DJ, Cao X. Neuropeptide Y is produced in visceral adipose tissue and promotes proliferation of adipocyte precursor cells via the Y1 receptor. Faseb J. 2008;22(7):2452-64.

8. Serradeil-Le Gal C, Lafontan M, Raufaste D, Marchand J, Pouzet B, Casellas P, et al. Characterization of NPY receptors controlling lipolysis and leptin secretion in human adipocytes. FEBS Lett. 2000;475(2):150-6.

NPY receptor mRNA expressions in subcutaneous and visceral adipose tissues of normal weight and obese humans

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Introduction

Neuropeptide Y (NPY) plays a central role in appetite regulation via its receptors Y1R, Y2R and Y5R. Peripheral NPY and NPY receptor expressions were reported in many tissues including adipose tissue. Previous studies showed that NPY increased fat proliferation via Y1R and Y2R and fat differentiation via Y2R receptor in rodents. However, NPY receptor expressions differing in normal and obese human subjects have not been studied.

Objective

This study aimed to compare NPY receptor mRNA expressions in subcutaneous and visceral adipose tissues in normal weight and obese humans.

Method

Subjects (n = 30) were recruited from female patients who underwent abdominal surgery. NPY receptor mRNA expressions in adipose tissues were measured by real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis.

Results

Y1 receptor expression in subcutaneous and visceral adipose tissues

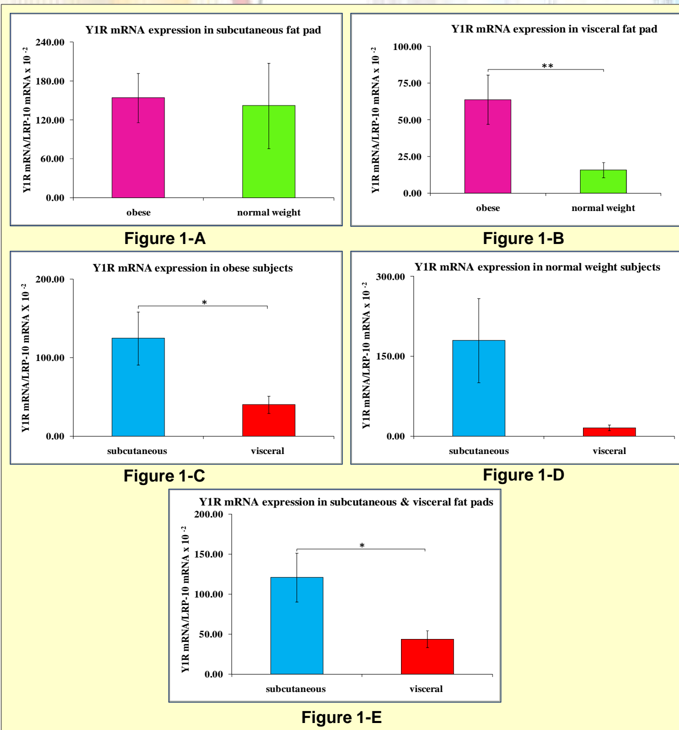

Figure 1

Y1R mRNA expression was higher in obese humans when compared to normal weight humans in visceral ($P<0.01$) but not in subcutaneous adipose tissues and was higher in subcutaneous adipose tissue of obese ($P<0.05$) and overall subjects ($P<0.05$) when compared to visceral adipose tissue.

Y2 receptor expression in subcutaneous and visceral adipose tissues

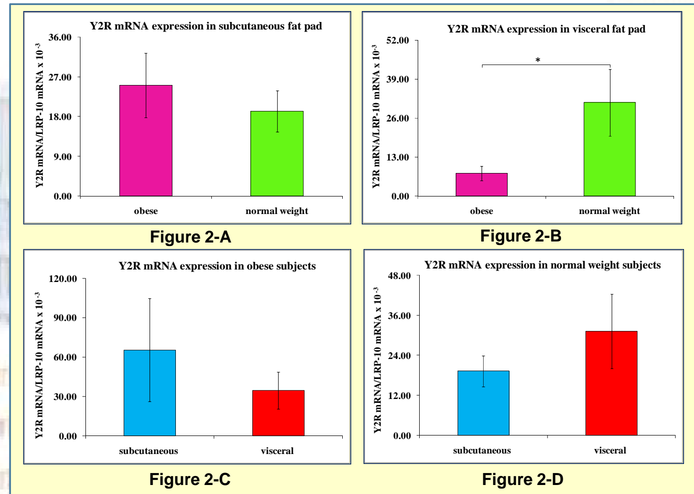

Figure 2

The Y2R mRNA expression was higher in normal weight subjects than obese subjects in visceral ($P<0.05$) but not in subcutaneous fat pad. There was no statistically significant difference between subcutaneous and visceral fat pads in obese and normal weight subjects.

Y5 receptor expression in subcutaneous and visceral adipose tissues

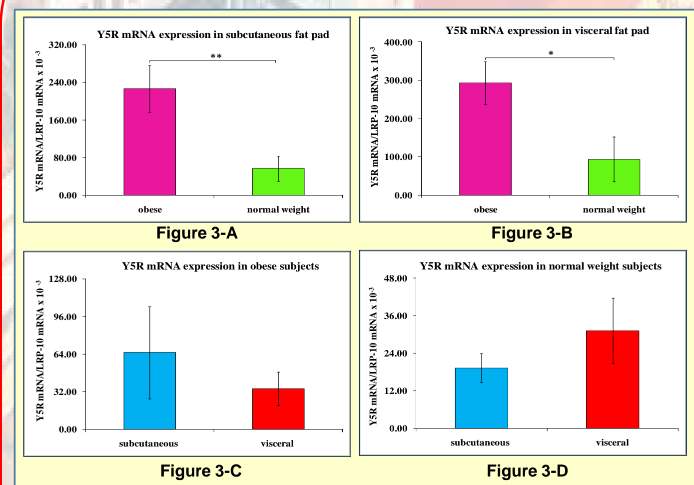

Figure 3

Y5R mRNA expression was higher in obese subjects in both subcutaneous ($P<0.01$) and visceral ($P<0.05$) fat pads than in normal weight subjects. Comparing Y5R mRNA expression between subcutaneous and visceral fat pads showed no significant difference in both obese and normal weight subjects.

Conclusion

- Y1R, Y2R and Y5R are differently expressed in adipose tissue in obese and normal weight humans with Y1R and Y5R being higher in obese subjects while Y2R being higher in normal subjects.
- Further investigation about blocking or activating Y1R, Y2R and Y5R receptors may propose new strategies for obesity treatment.