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Regulation of adipose tissue metabolism by NFkB P65 in transgenic mice

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REGULATION OF ADIPOSE TISSUE METABOLISM BY NFKB P65 IN TRANSGENIC MICE

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DEDICATION

This thesis is especially dedicated to my grandmother Yingshan Xiao, who passed away a few months ago. Your kindness and love will never be forgotten.

This thesis is also dedicated to my mother Meibin Li, my father Yaoming Tang, and my step father Suisheng Qian. It is because of you that I am the one today.

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ABSTRACT

Inflammation has been widely reported to regulate adipocyte functions in adipose tissue. Our early study suggests that NF-κB signaling pathway is activated by inflammation and involved in inhibition of insulin sensitivity in adipocytes. NF-κB was found to inhibit PPARγ function through several possible mechanisms in 3T3-L1 adipocytes. To test this possibility in vivo, we increased the NF-κB activity in adipocytes in transgenic mice by expression of NF-κB p65 subunit under the aP2 gene promoter.

The phenotype study shows that the food intake, physical activity and development are similar in the two groups. The reproductivity was not different in the two groups. However, the body weight gain and fat content increment are apparently less in the Tg mice, which was associated with a significant increase in energy expenditure and a defect in adipogenesis. Chronic inflammation was observed in the adipose tissue of Tg mice with macrophage infiltration and secretion of inflammatory cytokines. The data suggest that NF-κB p65 inhibits PPARγ function in adipose tissue, and prevents adulthood and diet-induced obesity. However, it does not provide benefit to the protection of systemic insulin sensitivity.

CHAPTER 1

INTRODUCTION OF BACKGROUND

OBESITY

Obesity has been a serious health threat to general public all over the world. A survey conducted by USCDC (United States Centers for Disease Control) estimated that 66% of the U.S. adult population are overweight, with 32.3% in obesity (body mass index (BMI) $\geq$ 30 kg/m2) [1]. Obesity may reduce life quality and lead to premature mortality through several chronic diseases. It was reported that the United States government has to spend $93 billion or 9.1% of all healthcare dollars on the disease associated with obesity [2].

It has been well accepted that people with abdominal obesity are at higher risk for heart disease, type 2 diabetes, hypertension and hyperlipidemia compared to people with subcutaneous obesity [3]. Although the mechanism for the health difference between visceral fat and subcutaneous fat remains unclear, literature suggests that visceral adipose tissue has a higher rate of lipolysis, which may promote elevation of non-esterified fatty acids (NEFAs) in the plasma, thus inducing very-low-density lipoprotein (VLDL) production, hepatic glucose output (HGO) and decreasing peripheral insulin sensitivity [4]. Therefore, the study of adipose tissue has been very active in the obesity research field.

ADIPOSE TISSUE FUNCTION

Adipose tissues are divided into white and brown types according to their cell types. The vast majority of white adipose tissue (WAT) volume is composed of lipid-laden adipocytes. But WAT also contains a significant stromovascular fraction (SVF), including preadipocytes, endothelial cells, smooth muscle cells, fibroblasts, leukocytes and macrophages[5]. The macrophage is the primary cell type in inflammation of adipose tissue. Activated macrophages

can secrete a plethora of cytokines such as TNF-α, IL-1 and IL-6 [6]. Several studies identified macrophages as the predominant producer for TNF-α and interleukin-6 in adipose tissue [7, 8]. In humans adipose macrophage density is positively correlated with BMI and insulin resistance [9] [10].

The major functions of WAT include energy storage and endocrine (secretion of hormones/adipokines). The hormones/adipokines control energy balance, glucose and lipid metabolism. The adipokines include leptin, adiponectin, resistin, tumor necrosis factor-alpha (TNF-α), plasminogen activator inhibitor-1 (PAI-1), MCP-1, macrophage migration inhibitory factor (MIF), interleukin-8 (IL-8), IL-6, IL-1 and vascular endothelial growth factor (VEGF).

Leptin acts mainly on the central nervous system to regulate food intake and energy expenditure. Leptin is also able to control TNF-α production and macrophage activation. It thus contributes to the limitation of inflammation states in obesity [11]. Adiponectin has multiple effects such as improving insulin sensitivity and protecting against atherosclerosis. Adiponectin can activate AMPK and enhance lipid oxidation [12]. Circulating adiponectin levels are decreased in obese and/or type 2 diabetic patients and in patients with coronary heart diseases. Serum resistin is elevated in obesity and involved in pathogenesis of insulin resistance. Resistin can also upregulate the expression of Suppressor of Cytokine Signaling-3 (SOCS-3), a well-known negative regulator of insulin signaling [13].

Brown adipocytes contain a high density of mitochondria, but less triglyceride. Their major function is to generate heat in the adaptive thermogenesis for maintenance of body temperature. Brown adipose tissue (BAT) is named after its color due to a high content of mitochondria. Upon cold stimulus, heat production in the animals is generated by BAT via the SNS-stimulated release of catecholamines. Such a process requires accelerated oxidation of

stored lipids within brown adipocytes in numerous mitochondria. Brown adipose cells exclusively express uncoupling protein-1 (UCP-1), a proton transporter that uncouples electron transport from ATP production, facilitating the energy to dissipate as heat. Based on the critical role of BAT in metabolism, BAT is now a hot point in the study of tumor and cancer [14, 15].

INFLAMMATION AND ADIPOSE TISSUE FUNCTION

Inflammation has been already detected with inflammatory cytokines such as TNF-α, IL-1 and IL-6 in expanded adipose tissue [6]. The expansion of WAT in obesity results in tissue hypoperfusion and hypoxia, resulting in macrophages infiltration and expression of inflammatory cytokines [16, 17]. The serum free fatty acids (FFAs) level is also elevated in obesity, which induces lipotoxicity and oxidative stress, thus inducing inflammation and impairing the insulin effect [18, 19]. Moreover, inflammatory mediators and lipid accumulation can affect the endoplasmic reticulum (ER), causing ER stress and enhancing inflammation [20, 21].

Obesity is also associated with increased numbers of apoptotic and necrotic adipocytes in WAT and BAT, which may induce chemoattractant signals that recruit monocytes[16]. There is evidence to suggest that macrophages alter inflammatory status in adipose tissue by promoting TNF-α, IL-6 secretion and ectopic storage of lipid within liver and muscle tissues [22]. As a result, enhanced secretion of some interleukins and inflammatory cytokines as well as increased circulating levels of many cytokines including TNF-α, IL-1, IL-6 and IL-8 happens in WAT and induces insulin resistance.

Tumor necrosis factor-alpha (TNF-α) is a multifunctional proinflammatory cytokine produced predominantly by monocytes/macrophages. It has been reported to be involved as an important regulatory cytokine in many cellular and biological processes such as cell

differentiation, proliferation, apoptosis, energy metabolism and immune function. The role of TNF-α in BAT is still debatable. Some studies supported that TNF-α can reduce mitochondrial biogenesis and function in WAT and BAT through inhibition of eNOS expression [23]. TNF-α also impairs BAT thermogenesis via downregulation of UCP-1 and beta 3-AR expression [24, 25]. It was reported that TNF-α could compromise BAT thermogenesis by promoting Brown cell atrophy or by impairing BAT differentiation or inducing BAT apoptosis [26, 27]. Conflicting opinions argue that TNF-α promotes BAT thermogenic activity, probably by increasing UCP proteins expression [28], but some studies on catabolic states tend to believe that TNF-α affects central nervous system to propel thermogenesis and energy expenditure [29].

As a contrast, there are numerous studies aiming at the effect of TNF-α on WAT. In diet-induced diabetic rodents, TNF-α is elevated in WAT and might mediate obesity-related insulin resistance [30]. In obese mice with a targeted null mutation in the genes for TNF-α and its p55 and p75 receptors, a significantly improved insulin sensitivity was observed in both diet-induced obesity and the ob/ob model of obesity due to the deficiency of TNF-α. TNF-α -deficient mice had lower levels of circulating free fatty acids and they demonstrated less obesity-related reduction in insulin receptor signaling in muscle and fat tissues. Furthermore, relevant experiments in vivo and in vitro supported that TNF-α might affect different sites of the insulin signaling pathway such as inhibiting of the insulin-stimulated tyrosine kinase activity of the insulin receptor, downregulating the expression of the insulin-sensitive glucose transporter Glut4 [31].

In addition to its effect in the local area, adipose tissue-derived TNF-α can regulate systemic energy homeostasis indirectly by affecting both adipose function and expandability. In the series of studies on catabolic disease, TNF-α was initially proved to play a role in cellular

metabolism. Mounting evidences show that TNF-α can affect glucose homeostasis in adipocytes[32], stimulates lipolysis[33] and inhibits adipocyte differentiation and lipogenesis.

Lipid metabolism in adipose tissue is composed of three main parts: FFA uptake, lipogenesis and lipolysis. TNF-α regulates lipid metabolism in adipocytes by inhibiting FFA uptake and lipogenesis, as well as promoting lipolysis. Lipoprotein lipase (LPL) is closely related to FFA uptake into adipocyte. The expression of LPL gene has been shown to be suppressed by TNF-α [34]. Although the levels of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), two proteins that mediate lipolysis, are downregulated when PPARγ activity is inhibited, TNF-α may still induce lipolysis by increasing cAMP concentration and impairing the preventative effect of perilipins on lipolysis [35]. These actions of TNF-α may be mediated via serine kinases JNK, ERK1/2 and NF-κB [36, 37]. The inhibition of PPARγ by TNF-α might also contribute to the decreased expression of perilipin [38]. Furthermore, fatty acid synthase (FAS) is downregulated by TNF-α, suggesting that lipogenesis might also be reduced in the presence of TNF-α [39].

TNF-α may also suppress adipogenesis by preventing the differentiation of preadipocytes into mature adipocytes, which is associated with the inhibition of PPARγ. TZD treatment can antagonize this effect [40]. And the expressions of other genes responsible for the function of adipocytes such as aP2, ATGL are also inhibited as a consequence of the downregulation of PPARγ and C/EBPα [41]. Another mechanism involved in the anti-adipogenesis effect of TNF-α is the β-catenin/TCF pathway. Activation of this pathway was confirmed to inhibit PPARγ expression and lipid accumulation both in vitro and in vivo [42].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor belonging to the nuclear receptor superfamily. PPARγ is essential in adipogenesis and

recognized as a major target for the insulin-sensitizing effects of the thiazolidinediones. Heterozygous PPARγ -deficient mice are protected from high-fat diet (HFD)-induced adipocyte hypertrophy, obesity and insulin resistance, suggesting that PPARγ may have a vital role in adipocyte hypertrophy, obesity and insulin resistance [43]. PPARγ is also one of the target genes of TNF-α [44]. TNF-α regulates PPARγ directly either by inhibiting the expression of PPARγ mRNA or suppressing its transcriptional activity. The TNF-α activity is dependent on activation of the nuclear factor kappa B (NF-κB) pathway [45].

NF-κB mediates a large amount of stimulis including the TNF-α signal in many immune or inflammatory conditions involving autoimmune arthritis, asthma, lung fibrosis, glomerulonephritis, atherosclerosis, Crohn's and insulin resistance. Inhibition of NF-κB salicylates was demonstrated to reverse insulin resistance [46]. After treatment with NF-κB inhibitors for 18 hours, production of IL-6, IL-8, and TNF-α was significantly reduced in adipocytes and skeletal muscle cells. And NF-κB p65 DNA-binding activity was also reduced. Moreover, NF-κB inhibitor increased the adipose tissue and skeletal muscle expression of insulin receptor-beta, suggesting NF-κB transcription pathway is a key regulator of IL-6, IL-8, and TNF-α [47]. Recently, more research has come to concentrate on the association between NF-κB and metabolism. Downregulation of NF-κB activity may attenuate the development of cachexia in tumor-bearing humans and mice through inhibition of IL-6 and TNF-α synthesis, suggesting the important role of NF-κB in metabolism [48-50].

In our previous studies, we have demonstrated that IKK inhibits the transcriptional activity of PPARγ through activation of NF-κB (Gao JBC 2006). Using multiple systems, we demonstrated that activation of NF-κB led to inhibition of PPARγ through several approaches. NF-κB inhibits mRNA expression of PPARγ and this happens after overnight NF-κB activation.

In an acute manner, activation of NF-κB leads to inhibition of the transcriptional activity of PPARγ through activation of HDAC3. In the cytoplasm, HDAC3 is associated with IκBα. After IκBα degradation in response to TNF-α, HDAC3 is subject to nuclear translocation, leading to a subcellular redistribution of HDAC3. HDAC3 is known as a component of corepressor complex for PPARγ [51]. In this case, the DNA-binding activity of PPARγ is not changed by NF-κB. Additionally, activation of IKK/NF-κB also promotes degradation of PPARγ protein. Our previous findings suggested that FFAs may use IKK/NF-κB pathway in the inhibition of PPARγ function [52, 53].

Moreover, our study in 3T3-L1 adipocytes strongly supported that the transcriptional coactivators and corepressors play a critical role in the inhibition of PPARγ by NF-κB. The κ transcriptional activity of PPARγ depends on recruitment of the transcriptional coactivators including SRC-1, SRC-2 and p300/CBP, which are also required by NF-κB [54]. Competition for a limited amount of the coactivators is a common mechanism of cross-inhibition between the two transcription factors, which might be the molecular basis that NF-κB deprives PPARγ of the transcriptional coactivators. However, we currently gained results only in vitro. The condition in vivo still needs further exploration.

The p50 (NF-KB1)/p65 (RELA) heterodimer is the most abundant form of the NF-κB complex, which is inhibited by IκB proteins. Phosphorylation of serine residues on the IκB proteins by kinases results in the degradation of the protein and thus allowing activation of the NF-κB complex [55]. Activated NF-κB complex translocates into the nucleus and binds DNA at kappa-B-binding motifs. The process requires the transcriptional coactivator CBP /p300 associating with NF-κB p65 subunit. Thus p65 serves as an essential functional subunit, which is supported by many studies. In the transgenic knockout mouse line for p65, the lack of the protein

led to embryonic lethality at 15-16 days of gestation due to massive degeneration of the liver due to apoptosis [56]. Some investigations reported direct evidence for the involvement of p65 in chronic intestinal inflammation in mice, suggesting that p65 antisense oligonucleotides might be a potential molecular treatment of patients with Crohn disease [57]. Furthermore, p65/RelA can directly bind to PPARγ-PGC1 complex and thus preventing it binding to PPARγ response elements[58]. Therefore, the concentrated study on NF-κB P65 may further illustrate the role of P65 in the function of NF-κB and its interaction with other subunits.

CHAPTER 2

PHENOTYPE STUDY OF AP2-P65 TRANSGENIC MOUSE

INTRODUCTION

Inflammation regulates adipocyte functions in adipose tissue. Our early study suggests that NF-κB signaling pathway is activated by inflammation and involved in inhibition of insulin sensitivity in adipocytes. Moreover NF-κB was found to inhibit PPARγ function through several possible mechanisms such as activation of nuclear corepressor and competition in coactivator in 3T3-L1 adipocytes. To test this possibility in vivo, we increased the NF-κB activity in adipocytes in transgenic mice. This was achieved by expression of NF-κB p65 subunit under the aP2 gene promoter.

The aP2-p65 transgenic mice (Tg mice) were utilized to study the phenotype and test the inflammation activity in adipose tissue. The Tg and WT mice had no difference in size, appearance and behavior at birth. The reproductiveity was not different in the two groups. The phenotype study in adult mice was started at the age of 6 weeks, focusing on the body weight, body composition, food intake, metabolic rate and adiposity. On chow diet, the food intake and physical activity were similar in the two groups. However, the body weight gain and fat content increment are apparently less in the Tg mice, which was associated with a significant increase in energy expenditure and a defect in adipogenesis. Chronic inflammation was observed in the adipose tissue of Tg mice with macrophage infiltration and secretion of inflammatory cytokines. The mRNA levels of PPARγ as well as its target genes were remarkably depressed. When fed on a high-fat diet, the Tg mice gained less fat and were protected from dietary obesity. The data suggest that NF-κB p65 inhibits PPARγ function in adipose tissue, and prevents adulthood and

diet-induced obesity. However, it does not provide benefit to the protection of systemic insulin sensitivity.

MATERIALS AND METHODS

Reagents: Rabbit polyclonal antibodies to NF-κB p65, p50 were purchased from Santa Cruz Biotechnology. The18s and p65 primers for genotyping PCR was purchased from Sigma-Aldrich Corporation. Taqman Primers and probes for p65 (Mm00501346_m1) were obtained from Applied Biosystems Incorporation. Real time RT-PCR reagents including MuLV reverse transcriptase, RNAse inhibitor and PCR reagent were obtained from Applied Biosystems (Branchburg, NJ).

Animals: Male C57BL/6J mice were purchased from the Jackson laboratory (Bar Harbor, ME). All of the mice were housed in the animal facility at the Pennington Biomedical Research Center with a 12:12-h light-dark cycle and constant temperature (22–24°C). The mice had free access to water and diet (normal chow MF 5015 consisting of 13.4% of calories as fat or a high fat diet D12331, Research Diets, New Brunswick, NJ), which contains 58% calories in fat.). All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center. The mice were ear-punched for identification and were kept four per cage.

aP2-p65 Constructs: The aP2-p65 plasmid construct was made by Gang Yu in the gene regulation lab at the Pennington Biomdical Research Center. To generate the aP2 promoter–P65 expression construct, a 1.8kb mouse p65 cDNA was amplified by PCR and was placed at the downstream of aP2 gene promoter in the aP2-PBSK vector at SpeI and Not I sites. The aP2-p65 expression cassette was then inserted into the Hind III/Not I sites in the multiple cloning region

of pcDNA3.1 (+) plasmid to gain poly A signal. The plasmid (20 g) was digested with Hind III (40 U) and Drd I (40 U) to isolate the aP2-p65-poly-A expression cassette. The digestion was conducted in 100 μl reaction with Buffer 4 overnight (16 hrs). The product was resolved in an agarose gel with 1X TAE buffer. The bands are shown in the picture. The DNA of aP2-PDGF-poly-A was the largest band, and was collected for making the transgenic mice. DNA sequence of all constructs was verified by DNA sequencing. The mouse p65 cDNA was obtained from the Dr. David Baltimore lab.

Generation of Transgenic Mice: The aP2-p65 transgene was microinjected into the pronuclei of fertilized mouse eggs taken from superovulated C57BL/6 F1 females. Injected embryos were implanted into the oviducts of surrogate females of the same genetic background. Transgenic founders were selected for breeding for new generations on a C57BL/6 background. New-born pups were weaned at 3 week age. Transgenic mice and wild-type (WT) littermate share identified with genotyping PCR, and WT littermates were used as controls. These procedures were conducted in the transgenic core facility at the Pennington Biomedical Research Center. The aP2-p65 mice (Tg mice) used for experiment are heterozygotes.

DNA Preparation and Genotype PCR: DNA was prepared from 0.5 cm tail sample at 3 week in age using the proteinase K protocol. The tissues were digested with proteinase K (FisherBiotech, Houston, TX) at 55 ^{o}C overnight. DNA (300ul) was purified with 0.5 ml phenol-chloroform and centrifuged at 14, 000 x g at room temperature. Cold ethanol (0.5ml 100%) was added to precipitate DNA. After washed with 75% ethanol and air dried, the DNA pellets were dissolved in 50ul DNase-free H_2O and stored at -20°C before genotype PCR was performed. The genotype PCR was performed with Alpha PTC DNA engine systems. The PCR primers were ordered from Sigma: Forward primer: GGACTTCTCTGCTCTTTTGAGTCAGA. Reverse

primer: CCTACTCAGACAATGCGATGCAA. The reaction mixture contained: 200ng DNA, 0.5ul thermol buffer, 50nmM NTP, forward primers, reverse primers and 2.5U Tagman enzyme. The reaction conditions were: 94°C for 5 min, 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec plus 33 cycles of 94°C for 45 sec, then 72°C for 5 min and 4°C forever. PCR products were running in 1% Agarose gel plus EB and final results were detected in a Bio-Rad ultraviolet detector.

RNA Preparation and Quantitative Real Time RT-PCR: Mice were sacrificed after a 6-h fast and the epididymal fat pad (for epididymal WAT), subcutaneous fat (for subcutaneous WAT), BAT, liver, and muscle were collected immediately and kept in liquid nitrogen. Total RNA was prepared using 1ml TRIzol Reagent (Invitrogen) for each sample according to the manufacturer's instructions. The homogenate-chloroform was mixed with 200 ul of chloroform and centrifuged at 18,000 x g at 4°C for 15 min for phase separation. For RNA precipitation, 300ul of aqueous supernatant was transferred to a 1.5 ml-Eppendorf tube, mixed with 500ul of isoproponal, incubated at room temperature for 10 min, and then centrifuged at 18,000 x g at 4°C for 15 min. The precipitated RNA pellet was washed by 500 ul of 75% ethanol, centrifuged at 18,000 x g at 4°C for 5 min, the ethanol was removed, and the RNA pellet was allowed to dry in air for 5 to 10 min. The RNA pellet was dissolved in 20ul of RNase-free H_2O and stored at -80°C. p65 expression was examed by real time RT-PCR. 18s was used as an internal control. Real time RT-PCR was performed on an ABI PRISM 7700 sequence detector. The reaction mixture contained: 15 ng RNA, PCR buffer, 5.5 mM $MgCl_2$, 5 U RNAse inhibitor, 12.5 U MuLV Reverse Transcriptase, 1.5 U AmpliTaq Gold DNA polymerase, dATP, dCTP, dTTP and dGTP each 0.3 mM, 500 nM forward primers, 500 nM reverse primers, 200 nm Taqman probes, (Applied Biosystems). The reaction conditions were: 48°C for 30 min for RT; one cycle of 95°C

for 10 min plus 40 cycles of 95°C for 15 sec and 60°C for 1 min for PCR. PCR products were sequenced. The relative level of mRNA was normalized to constitutive expression of 18s mRNA. Data are reported as means ± SE.

The primers used in the phenotype study of Tg mice were all from Applied Biosystems, including ACDC (Mm00456425_m1), aP2 (Mm00445880_m1), leptin (Mm00434759_m1), IL-6 (Mm00446190_m1), F4/80 (Mm00802530_m1), TNF-α (Mm00443258_m1), PPARγ (Mm00440945_m1), FAS (Mm00662319_m1), SREBP (Mm00550338_m1), SCD1 (Mm00772290_m1), LPL (Mm00434770_m1), HSL (Mm00495359_m1), MCP-1(Mm00441242_m1), IkBa (Mm00477798_m1), IL-10 (Mm00433859_m1), IL-18 (Mm00434225_m1), CD11c (Mm00434455_m1), Pref-1 (Mm00494477_m1).

Adipogenesis Study in Vitro: In this study, we collaborated with Dr. Barbara Kozak and Dr. Jaroslaw Staszkiewicz. Mice at 5 weeks in age were used for collection of the preadipocytes in the adipogenesis study. Each group had 5 mice. Their epididymal fat and inguinal fat were collected, minced and digested with collagenase type I (2 mg/1 ml; Worthington Biochemical, Freehold, NJ) in a shaking bath for 1h at 37°C. The cell suspension was filtered through a 70 μm cell strainer (Becton Dickinson Labware, NJ) followed by centrifugation (360 × g, 5 min, RT). Pelleted cells were resuspended in 1 ml red blood lysis buffer (Sigma Co., St. Louis, MO) and centrifuged as above. The isolated cells were plated in 100 mm Petri dishes (p = 0) in Dulbecco's Modified Eagle Medium DMEM/F12; Invitrogen, Carlsbad, CA) supplemented with 1% antibiotic solution and 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). Subconfluent primary cultures were trypsinized (0.05% trypsin/0.53 mM EDTA; Life Technologies, New York, NY) followed by immunomagnetic cell sorting. For *in vitro* adipogenic differentiation, cells were replated in 12-well culture plates (Corning, Corning, NY) at the density of 10^5/well

and maintained in complete medium until confluent (considered as a day 0). Thereafter, the cells were exposed to an adipogenic medium containing DMEM/F12, 5% FBS, 1% antibiotic solution, 0.5 mM isobutylmethylxanthine, 1.7 μM insulin and 1 μM dexamethasone for 2 days. For the 6 days of adipogenesis, the culture was collected at days 0, 2, 3, 6 and 8 for RNA purification.

Preparation of Whole Cell Extracts: Tissue samples were rinsed with PBS and then harvested in a nondenaturing buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Samples were sonicated for 3x6 times on ice and centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer's instructions. Supernatants were stored at -20 °C prior to Western immunoblotting.

Western Immunoblotting: Homogenates were separated in 8% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and transferred to PDVF membranes in 25 mM Tris, 192 mM glycine, and 20% methanol. After the transfer, the membrane was blocked in 4% milk for 30 min and incubated with the primary antibodies p65 (#P3363, Santa Cruz, MA). The signal was visualized with horseradish peroxidase-conjugated secondary antibodies from Sigma. Tubulin (#4699-9555, Biogenesis, Kingston, NH) was used as an internal control.

Nuclear Magnetic Resonance: Body composition was measured using quantitative nuclear magnetic resonance (NMR) as described previously [59]. In the test, conscious and unrestrained mice were placed in a small tube individually and the tube was inserted into a Minispec Mn10 NMR scanner for diction of body composition (Brucker, Canada, Milton ON, Canada). The fat and lean mass were recorded within 1 min.

General Follow-up and Blood Glucose Analysis: Follow-up of body weight and body composition of the mice were performed every two weeks. The mice were fasted overnight and physically examined the next morning. The body compositions were detected as described above. The relative percent of body fat content and muscle content were adjusted according to individual body weight. Blood glucose concentrations were measured with a One-touch blood glucose monitor (Freestyle, Abbott) every four weeks. Whole blood was collected with heparinized capillary tubes (Fisherbrand, PA) and centrifuged at 4 °C at 4000 X for 20 min to obtain serum samples. Serum samples were individually labeled and kept at -20°C before testing. At the age of 16 weeks, the diet of the mice was switched from a chow diet to a high-fat diet and the follow-up went on as described before. Body weight and blood glucose results are presented as an average ± SD.

Food Intake: Food intakes of mice were evaluated to determine whether there is any difference between WT mice and Tg mice. The test lasted for 3 days. Mouse body weights prior to and after the test were measured. At the beginning, food weight was measured and at the end of the test, left food not eaten was carefully collected and weighed. The difference of food weight prior to and after the test adjusted by body weight stands for the food intake of the mice. Results are presented as an average ± SD.

Indirect Calorimetry: Indirect calorimetry measurement was performed with Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH) in the Pennington Biomedical Research Center. The experiment lasted for a week, during which mice were free to consume food and water. The mice were housed individually in the metabolic chamber. Ambient room temperature ranges between 23 and 25°C. Only parameters obtained in steady-state conditions were used for final calculations and evaluations (steady state refers to the

state as ≥3 min of VO_2 within 10%, of respiratory quotient within 5%, and of air flow through the canopy system within 10%). After 48 h of adaptation, the data involving energy expenditure, respiratory exchange ratio (RER), and spontaneous physical movement were simultaneously recorded for each individual mouse and used in analysis of the metabolic phenotype.

Intraperitoneal Insulin Tolerance (ITT): ITT was conducted by intra-peritoneal (i.p.) injection of insulin (I9278, Sigma) at 0.75 U/kg body weight in mice after a 4 hour fast as previously described (Gao et al., 2007). Blood glucose was monitored in the tail vein blood using the FreeStyle blood glucose monitoring system (TheraSense, Phoenix, AZ).

Glucose Tolerance Test (GTT): For glucose tolerance tests, mice were fasted overnight, and glucose (2.5 g/kg body wt) was administered intraperitoneally the next morning. Then tail vein blood glucose concentrations were measured at 0 min, 30 min, 60 min, 120 min and 180 min with Freestyle One-touch glucose monitor (TheraSense, Phoenix, AZ).

Immunohistological Staining: Fresh epdidymal fat and liver tissue were collected and fixed in fresh 4% formalin for at least 24 hrs, then washed in Phosphate Buffered Saline (PBS) buffer for 2 hrs. After mounting dehydrated in 70%, 95% and 100% ethanol and treated with xylene, the tissues were embedded into melted paraffin. The tissue slides were obtained through serial cross-section cutting at 5 um thickness and processed with a standard procedure including dewaxed with xylene, hydrated with 100%, 95%, 80% and 70% step by step, treated with 0.2% H2O2, antigen unmasked in 0.01M sodium citrate buffer, treated with 0.2% Triton-x-100, and incubated with goat serum diluted in 1%BSA. The slides were then blotted with a monoclonal primary antibody of F4/80(sc-71087, Santa Cruz) at 1:200 dilution. After being washed with PBS, the slide was incubated with a biotinylated secondary antibody (mouse IgG) in ABC kit. The slides were then incubated with the ABC elite reagent (PK-6101) and color reaction was

performed using the DAB substrate kit (SK-4100) for myosin I and AEC substrate kit (AEC101, Sigma) for F4/80 according to instructions by the manufacturers.

Preadipocyte Adipogenesis Study: The experiment was done with the collaboration of Drs. Barbara Kozak and Jaroslaw Staszkiewicz. The detail methodology has been described in the paragraph of adipogenesis study in vitro in chapter 2.

Fluorescent Staining: Differentiated cells were fixed with 10% formalin for 1 h at RT followed by staining with 300 nM DAPI 10 µg/ml BODIPY® 493/503 (Invitrogen, Carlsbad, CA) in PBS for 20 min at RT. After washing 3 × 5 min in PBS, cells were imaged with a Plan Fluor DL 10× objective using a Nikon Eclipse TE2000-U inverted microscope equipped with a CoolSnap camera. Images of random areas were captured and stored with Metamorph imaging software. Fluorescence was measured on a FLEXstation Benchtop Scanning Fluorometer (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 490 & 545 nm (Bodipy), and 355 & 450 nm (Dapi), respectively. Results are presented as a mean ratio BODIPY/DAPI ± SD.

Primary Macrophage Study: Primary peritoneal macrophages were isolated from Tg mice and WT littermate of 24-week-age, as what we described before[59]. The macrophages were induced intraperitoneally by injection of 2 ml of sterilized solution of 2% starch (Sigma 85643). The macrophages were harvested 3 days later with 20 ml of cold PBS in lavage and then cultured in DMEM (supplemented with 10% FBS and 50µg/ml gentamicin) in a 100-mm culture dish. Three days later, the cells were transferred to 35-mm tissue culture dishes and treated with or without LPS (2ng/ml, Sigma) in serum-free RPMI 1640 medium. 2 hrs later, median was removed and cell RNA was collected and purified.

Statistical Analysis: Statistical analysis for food intake, body weight, body content,

indirect calorimetry, fasting blood glucose, ITT, GTT, blood lipid profile, blood cytokine level and all real time RT-PCR in tissues was performed using two-tailed unpaired Student's t-test for WT littermates versus Tg mice.

RESULTS

Generation of Transgenic Mice: The construction of aP2-p65 plasmid is illustrated in Fig. 1A. One male and two female founders of the Tg mice were born in Dec. 26, 2006 and delivered to our lab from the transgenic core facility. The genotype PCR was conducted in all of the three founders and their positivity was confirmed. Transgene was indicated by a PCR product of 400bp in the tissue of positive mice. The result from founder 2 is presented in Fig. 1B. All of the founders were used to breed with C57/B6 WT mice to amplify each colony. The founder 2 was used in the following study for its productivity in breeding. In this study, male transgenic (Tg) mice were used in the following studies. Age-matched WT littermates were used as the control.

p65 Expression in the Adipose Tissue of Transgenic Mice: To confirm p65 expression in adipose tissue in the transgenic mice, p65 mRNA levels were examined in different tissues, and the expression was compared between Tg and Wt mice. In the study, samples were collected from Tg and WT mice with 6 in each group. The results from qRT-PCR showed that the p65 mRNA level was significantly elevated in adipose tissue of Tg mice. The elevation was observed in both WAT and BAT, but not in other tissues such as liver. In the Tg mice (Fig 2 A, B, and C), the p65 protein level was also elevated in the adipose tissue. These results demonstrated that NF-κB p65 overexpression is achieved specifically in the adipose tissue of Tg mice.

p65 Expression in Adipogenesis: Preadipocytes were isolated from the inguinal fat pad and used in vitro in a differentiation assay. Three mice were used in each group. Day 0 was the

starting point and day 6 was the peak point of differentiation. Expression of p65 was determined by mRNA and compared between Tg and WT cells. At day 0, there was no difference in p65 mRNA levels in the two groups (Fig 2F). After differentiation, the p65 mRNA level was 4 fold higher in the Tg cells, suggesting that the aP2 promoter activity was activated in mature adipocytes and this leads to expression of the recombinant p65 in mature adipocytes.

Energy Metabolism: To evaluate the energy metabolism, we tested the energy expenditure in the mice. In our study, energy expenditure was induced by overexpression of NF-κB p65 in the adipose tissue. This led to prevention of diet-induced obesity. The energy expenditure was studied in the mice on both chow and high fat diet (HFD). Food intake, physical activity, energy expenditure, oxygen consumption and substrate utilization were monitored using the metabolic chamber. There was not a significant difference in food intake and spontaneous physical activity on the chow diet between the two groups (Fig. 3, A and B). However, energy expenditure and oxygen consumption were increased in the Tg each group mice during both day and night time (Fig. 3, C and D). The fold increase in energy expenditure was much higher at night in the Tg mice (Fig. 3C).

On the high-fat diet, the food intake in Tg mice was even higher than that of WT mice (Fig. 4A). But energy expenditure was also increased in the Tg mice (Fig. 4, C and D). However, the fold increase was smaller at night time. The RER value was not changed in the Tg mice in chow or HFD (Fig. 3E and 4E), suggesting that substrate utilization was not changed in the Tg mice. This group of data suggests that p65 over expression in adipose tissue leads to an increase in energy expenditure in transgenic mice.

Body Weight: The body weight was recorded every two weeks in the study from 6 weeks in age when the Tg had the same body weight as the Wt mice. From 6 to 16 weeks on the

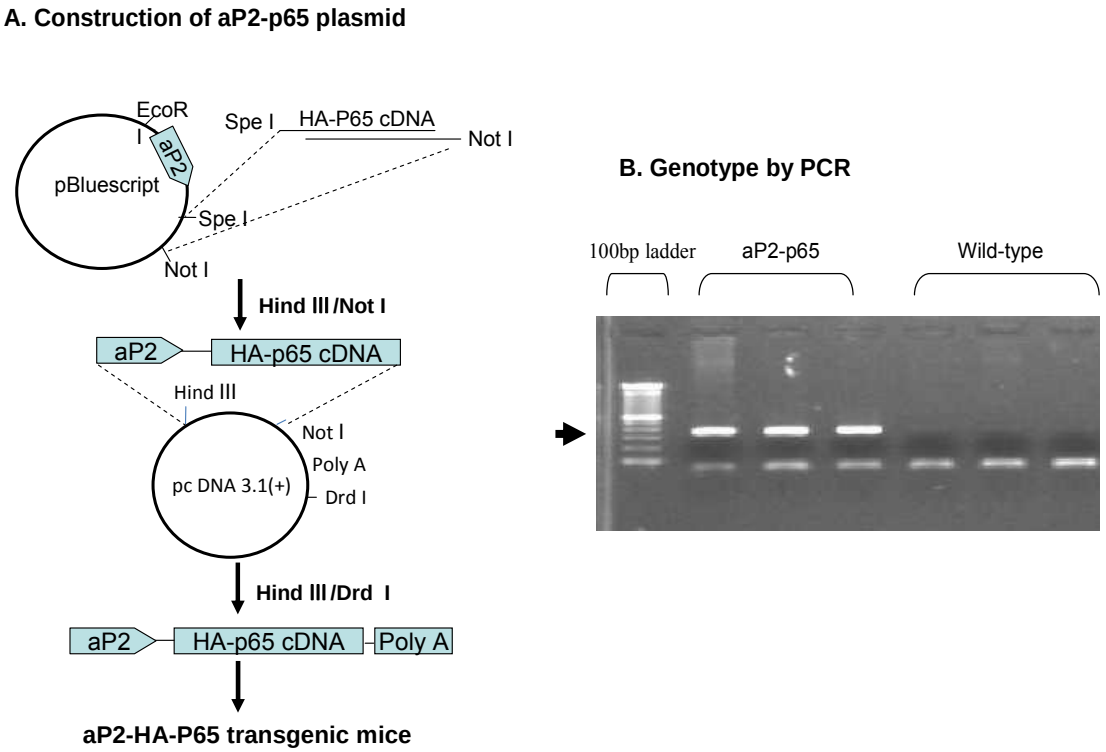

Figure 1. Generation of aP2-p65 transgenic mice (Tg mice). (A) The construction of plasmid vector for aP2-p65 expression cassette. The mouse p65 cDNA was obtained through PCR over a vector template (from Dr. David Baltimore lab). The PCR product was cloned into a Bluescript plasmid at the downstream of the aP2 (FABP4) gene promoter at Spe I/Not I sites. The aP2-p65 expression cassette was then inserted into pcDNA3.1 plasmid at Hind III/Not I sites to gain the Poly A signal. After confirmation by DNA sequencing, the aP2-p65-poly A fragment was purified and used for production of the transgenic mice in C57BL/6J strain. The transgenic mice were made in the core facility at PBRC; (B) Genotype PCR image of the aP2-p65 transgenic mice. At the age of 3 weeks, the pups were weaned and their tail tissue was collected to extract DNA. Transgenic gene was confirmed by genotype PCR with a PCR product of 400bp size.

chow diet, average body weight was increased by 66% (21 g to 35 g) in the WT mice (Fig. 3F), fat content (adiposity) was increased by 160% (10% to 26% BW) (Fig. 3G), and the percentage of lean mass was reduced by 20% (80% to 64%) (Fig. 3H). In the Tg mice, the average body weight was only increased by 5% (20 g to 21 g), fat content was increased 78% (9% to 16% BW) (Fig. 3G), and the percentage of lean mass was reduced by 12% (82% to 72%) (Fig. 3H). These data suggest that p65 is able to prevent adipose tissue growth in adulthood, and protect the Tg mice from adulthood obesity.

The phenotype study was conducted on HFD in mice, which were fed on HFD at 16 weeks in age. In the WT mice, average body weight was increased by 48% (Fig. 4F), fat content (adiposity) was increased by 59% (Fig. 4G), and the percentage of lean mass was reduced by 21% on HFD for 10 weeks (Fig. 4H). In the Tg mice, changes in this group of parameters are much smaller. The increases were 22% (32 g to 39 g) in body weight and 44% (16% to 23%) in the adiposity. The decrease was 8.2% (73% to 67%) in the lean mass, suggesting that adipose tissue specific overexpression of p65 attenuated weight gain and decreased adipose tissue expansion induced by high-fat diet. Mouse growth was not influenced by p65 overexpression as the body length was identical between the two groups (Fig. 3I). These data suggest that p65 is able to prevent adulthood obesity, and protect the Tg mice from diet-induced obesity. The increase in energy expenditure may be responsible for the phenotype in body weight.

Insulin Sensitivity: In the Tg mice, the lean phenotype may increase insulin sensitivty. To test this possibility, insulin sensitivity was examined by ITT. The fasting glucose was not changed in the Tg mice from 6 to 16 weeks in age (Fig. 5A). At 14~15 weeks in age, an insulin intolerance test (ITT) was conducted after 4 hr fasting. The Tg and Wt mice did not exhibit a significant difference in the test although there was a big difference in their body weight.

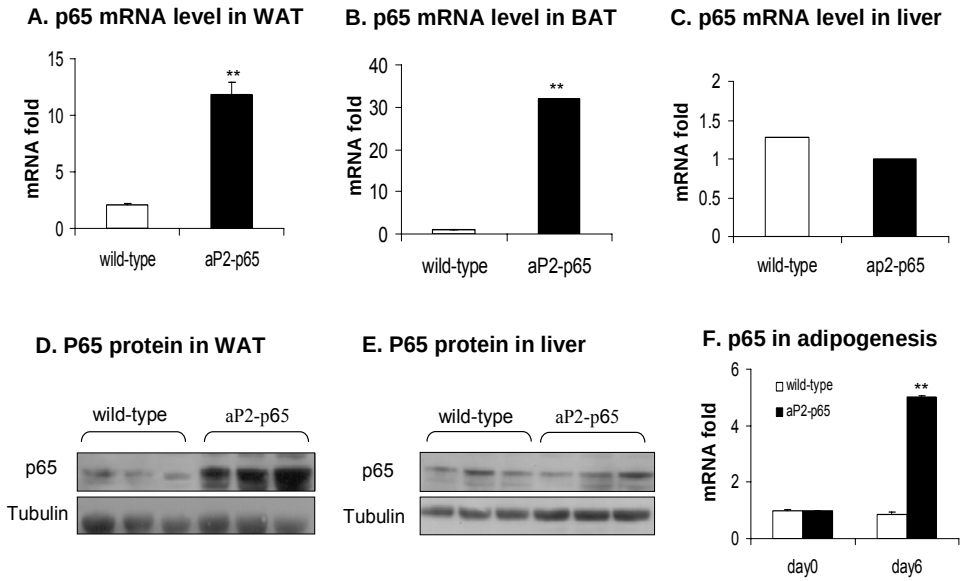

Figure 2. p65 expression level in different tissues. (A, B, C and F) Real time RT-PCR was performed to test p65 mRNA level in white adipose tissue, brown adipose tissue, liver and in adipogenesis study. (D, E) P65 protein expression in different tissues demonstrated with Westernblotting test. (A) Relative p65 mRNA level in white adipose tissue; (B) Relative p65 mRNA level in brown adipose tissue; (C) Relative p65 mRNA level in liver; (D) P65 protein expression in white adipose tissue; (E) P65 protein content in liver; (F) Relative p65 mRNA level in adipogenesis study in vitro. Preadipocytes were isolated from inguinal fat pad of 5-week-old mice. N=3 mice for Preadipocytes were induced differentiation after confluence. At day 0 and day 6, RNA were extracted from some cells. p65 mRNA level was determined by real time RT-PCR. For A-C, n=6 in wild-type (WT) or transgenic (Tg) group. For F, n=3 in each group, Values are the mean ± SE. *P<0.05, **P<0.001 by Student's t test.

No difference was detected between the two groups of mice on either chow diet or high-fat diet (Fig. 5 B and C). This result was unexpected and will be discussed later.

Glucose Tolerance: We also conducted the glucose tolerance test in the Tg mice to evaluate the pancreatic function. The study was conducted on the chow diet. Blood glucose levels were recorded at 0min, 30min, 60min, 120min and 180min after injection of glucose. After quantification of area under the curve, we found no difference between the two groups of mice (Fig 5, D and E). The data suggest that insulin secretion was not changed in the Tg mice.

WAT: The reduced adulthood adiposity in the Tg mice led us to examine structure and function of WAT. The epididymal fat pad was used in the analysis of adipocyte size and gene expression. The fat pad and adipocyte size were both reduced in the Tg mice (Fig. 6, A and B). The fat pad was 60% less in mass and adipocyte size was 40% smaller in the Tg mice. In gene expressions, mRNA was quantified by qRT-PCR in the adipose tissue. In the Tg tissue, adipocyte markers such as adiponectin and leptin were more than 50% lower than those in WT (Fig. 6C). The leptin mRNA was only 20% of that in the WT tissue. The aP2 and PPARγ mRNA was also much lower. The fat synthesis-related genes, FAS and SREBP were about 50% lower (Fig. 6D). These data suggest that fat synthesis and storage are decreased in the Tg mice. However, the genes related to lipolysis such as LPL and HSL were also reduced in Tg tissues (Fig. 6E). The data suggest that P65 overexpression is able to reduce triglyceride accumulation in adipocytes and the mechanism is related to inhibition of PPARγ function.

As expected, inflammatory cytokines, such as TNF-α, IL-1, IL-6, and MCP-1, were remarkably elevated by p65 in adipose tissue (Fig. 6F). Macrophage infiltration into the adipose tissue was also increased in the Tg mice. mRNA of Macrophage markers, such as CD11 and

F4/80, were elevated (Fig. 6G). F4/80 protein was increased in the adipose tissue of Tg mice (Fig. 6B). The inflammation may contribute to the inhibition of PPARγ function in the adipose tissue.

Adipogenesis in Vitro: The reduced PPARγ activity was associated with the lean phenotype in the Tg mice. According to our previous study, the PPARγ inhibition may be a direct consequence of p65 over expression in adipocytes. However, inflammation in the adipose tissue may also contribute to the inhibition. To test the direct effect of p65 in adipocytes, we conducted an adipogenesis assay in vitro using primary preadipocytes. The preadipocytes were isolated from inguinal fat and differentiated in the presence of adipogenic cocktail containing Pioglitazone. The differentiation was monitored by gene expression and TAG accumulation. In the Tg cells, expression of preadipocyte marker Pref-1 was not changed before differentiation, but significantly higher after differentiation compared to WT cells (Fig. 7A).

Adipokines such as adiponectin and leptin were not detectable in the preadipocytes, and their expression was 50% lower in the Tg cells after differentiation (Fig. 7, B and C). In differentiated cells, TAG abundance was 50% lower in the Tg cells (Fig. 7, D and E). The data suggest that preadipocyte number was not reduced in the Tg mice, but their potential in adipogenesis was decreased compared to WT cells.

The PPARγ mRNA was significantly lower in the Tg preadipocytes, and the difference was further increased after differentiation (Fig. 7F). The PPARγ target gene aP2 was also significantly lower in the Tg cells after differentiation (Fig. 7G). This is consistent with reduction in adiponectin (PPARγ target gene) expression (Fig. 7B). These data suggest that the PPARγ suppression may be responsible for the reduced differentiation potential in the preadipocytes of Tg mice. Since p65 expression was increased by several fold in the Tg cells after differentiation (Fig. 2F), the PPARγ inhibition is likely a result of the p65 overexpression.

Expression of inflammatory cytokines may not be required for the PPARγ inhibition in this system.

Inflammation in Other Tissues: To determine the tissue specificity of inflammation from p65 overexpression, we also tested gene expressions in brown adipose tissue, liver and skeletal muscle. Similar to the white fat, the brown fat exhibited an increase in mRNA for TNF-α, IL-1 and IL-6 in the Tg mice (Fig. 8A). Macrophage chemoattractant protein 1 (MCP-1) and macrophage marker F4/80 were also increased in the brown fat (Fig. 8B). Unlike in the adipose tissues, the liver and muscle did not exhibit an increase in inflammatory genes (Fig. 8, C and D). In combination with data in Fig. 2, these data suggest that inflammation was increased specifically in the adipose tissue of Tg mice.

DISCUSSION

P65 and Metabolism: The current concept about NF-κB in the regulation of metabolism is derived from evidence of IKK2 transgenic mice [60] and cellular models in which NF-κB activity is modulated by TNF-α or NF-κB related genes [51].

The exact function of NF-κB in adipose tissue has not been demonstrated in transgenic mice. In our study, we successfully established the transgenic mouse, and then characterized the metabolic phenotype of the mice. The results suggest that P65 overexpression in adipose tissue is able to increase whole body energy expenditure. The increase is able to prevent adulthood obesity, and protect the mice from dietary obesity. The energy expenditure is not from a change in physical activity or food intake. The reproductivity and development of the Tg mice was normal.

The mechanism for the lean phenotype was associated with inhibition of PPARγ activity in the Tg mice. Our data demonstrate that expression of the recombinant p65 is dramatically

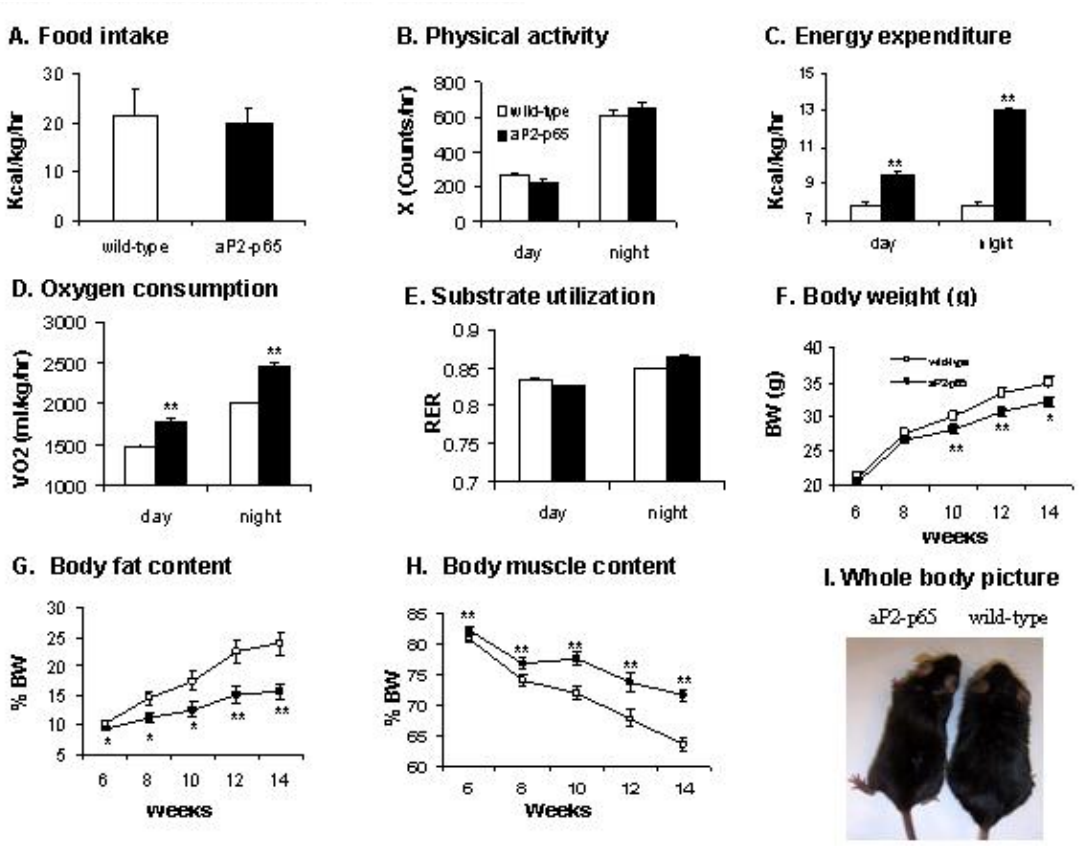

Figure 3. Energy metabolism in Tg mice and the WT mice on chow diet. P65 overexpression in WAT increased energy expenditure in aP2-p65 C57BL/B6 mice. Food intake and energy expenditure were examined using the metabolic chamber (12 weeks in age). (A) Food intake. Food intake was monitored daily for 3 days. Average daily food intake (g) was converted into K calorie and normalized with body weight (kg) and 24 hours; (B) Spontaneous physical activity. The frequency of horizontal movement was shown for day time and night time; (C) Energy expenditure. The unit is K caloric per kilogram body weight every hour; (D) Oxygen consumption. The unit is ml volume oxygen in kilogram body weight per hour; (E) Substrate utilization, expressed by respiratory exchange ratio (RER), which is a volume ratio of oxygen consumed versus CO2 exhaled; (F) Body weight; (G) Body fat content in percentage of body weight. This was determined by NMR; (H) Body muscle content in percentage of body weight; (I) Picture of experimental animals. It was taken on chow diet (at 20 weeks in age). For A-E, n=8, For G-H, n=10 in WT or Tg group. Values are the mean ± SE. *P<0.05, **P<0.001 by Student's t test.

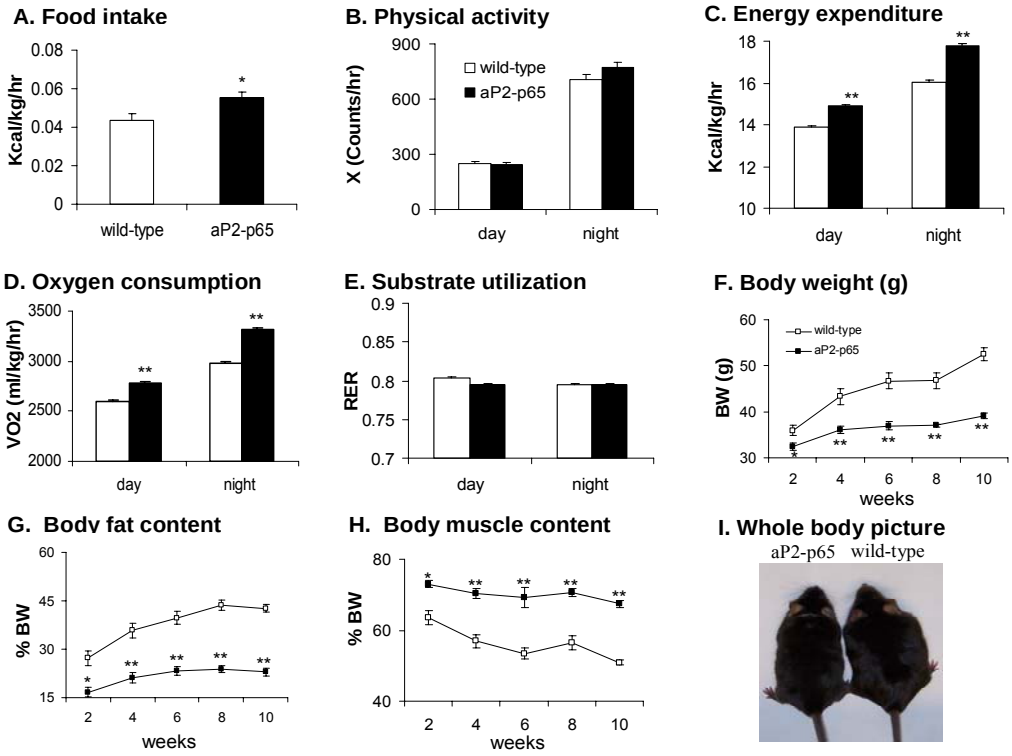

Figure 4. Energy metabolism in Tg mice and the WT mice on HFD. P65 overexpression in WAT increased energy expenditure in aP2-p65 C57BL/B6 mice. Food intake and energy expenditure were examined using the metabolic chamber (12 weeks in age). (A) Food intake. Food intake was monitored daily for 3 days. Average daily food intake (g) was converted into K calorie and normalized with body weight (kg) and 24 hours; (B) Spontaneous physical activity. The frequency of horizontal movement was shown for day time and night time; (C) Energy expenditure. The unit is K caloric per kilogram body weight every hour; (D) Oxygen consumption. The unit is ml volume oxygen in kilogram body weight per hour; (E) Substrate utilization. This is expressed by respiratory exchange ratio (RER), which is a volume ratio of oxygen consumed versus CO2 exhaled; (F) Body weight; (G) Body fat content in percentage of body weight. This was determined by NMR; (H) Body muscle content in percentage of body weight; (I) Mouse picture. It was taken on HFD for 10 weeks (at 26 weeks in age). For A-E, n=8, For F-H, n=10 in WT or Tg group. Values are the mean ± SE. *P<0.05, **P<0.001 by Student's t test.

Increased in mature adipocytes, but not in the preadipocytes. This pattern of expression is expected and is responsible for the inhibition of PPARγ function in the mature adipocytes. In addition to adipocytes, expression of the p65 is increased in macrophages, and this may contribute to the elevated activity in macrophages. In adipose tissue, the inflammation is increased as indicated by macrophage infiltration and inflammatory gene expression.

The inflammation may contribute partially to the inhibition of PPARγ function by promotion of NF-κB activity. TNF-α expression is increased in the adipose tissue, and it may inhibit adipocyte function through targeting PPARγ-responsive genes such as LPL [34], and perilipins [35].

P65 and Adipogenesis: In this study, we found that the body fat mass of Tg mice is much less than WT mice both on chow diet and high-fat diet. The size of adipocytes from Tg mice are 40% smaller. Furthermore, the expression of PPARγ and its target genes (aP2, and adiponectin) are decreased in adipose tissue and differentiated adipocytes from Tg mice. PPARγ is essential in adipogenesis, which has been showed in in vitro preadipocyte experiments[61] and the animal model of adipose tissue specific knockout of PPARγ [62]. The study of preadipocyte differentiation in vitro showed that preadipocyte number is not reduced in the Tg mice, but their differentiation was decreased. Gene analysis shows that the baselines of aP2, PPARγ, adiponectin, leptin and pref-1 are similar in the two groups before differentiation. However, after differentiating, all of the genes are lower in the aP2-p65 cells. The mRNA level of pref-1, which is mainly expressed in preadipocyte, was increased in the Tg cells after differentiation (Fig 7 C). A high level of pref-1 is connected with less adipogenesis[63], These data support that the adipose tissue specific overexpression of NF-κB P65 inhibited preadipocyte differentiation into mature adipocytes mainly through PPARγ inhibition.

P65 and Insulin Sensitivity: In our study, Tg mice have less possibility to gain weight and body fat mass on both chow diet and high-fat diet. Their adipocyte size is also significantly smaller than WT adipocytes. According to the phenomenon, we had expected to see better insulin sensitivity in Tg mice than their WT littermates. But we observed no meaningful improvement in insulin sensitivity either on chow diet or on high-fat diet, which was determined with fasting blood glucose levels and an insulin tolerance test. The results indicate that even though Tg mice did not develop obesity, the residential chronic inflammation in the adipose tissue plays a great effect on the systemic insulin effect. The Tg mice had an increased amount of adipose tissue macrophages with higher IL-6 and TNF-α levels. According to the findings in aP2-MCP-1 transgenic mice[64], we hypothesize that both the paracrine and the endocrine effects of inflammatory cytokines contributed to the development of insulin resistance in Tg mice. TNF-α and IL-6 might work locally on adipocytes, and skeletal muscle and the liver, causing alterations in metabolic and endocrine functions. In local adipocytes, increased lipolysis and decreased FFA uptake lead to elevated release of NEFAs, contributing to the exacerbation of insulin resistance in Tg mice. Furthermore, TNF-α and IL-6 may block the classic insulin signaling pathway through inhibition of phosphorylation of IRS proteins.

However, it is difficult to quantify the degree to which each of these effects contributes to the development of insulin resistance yet. Besides, PPARγ, adiponectin and leptin mRNA levels were also reduced in the WAT of Tg mice, suggesting that the anti-insulin resistance mechanism is impaired too. Therefore, our future research may focus on the insulin signaling pathway in adipose tissue.

A recently published study might provide another explanation for this phenomenon. The aP2-Lepr-b transgenic mouse model was used as an adipose tissue specific db/db model.

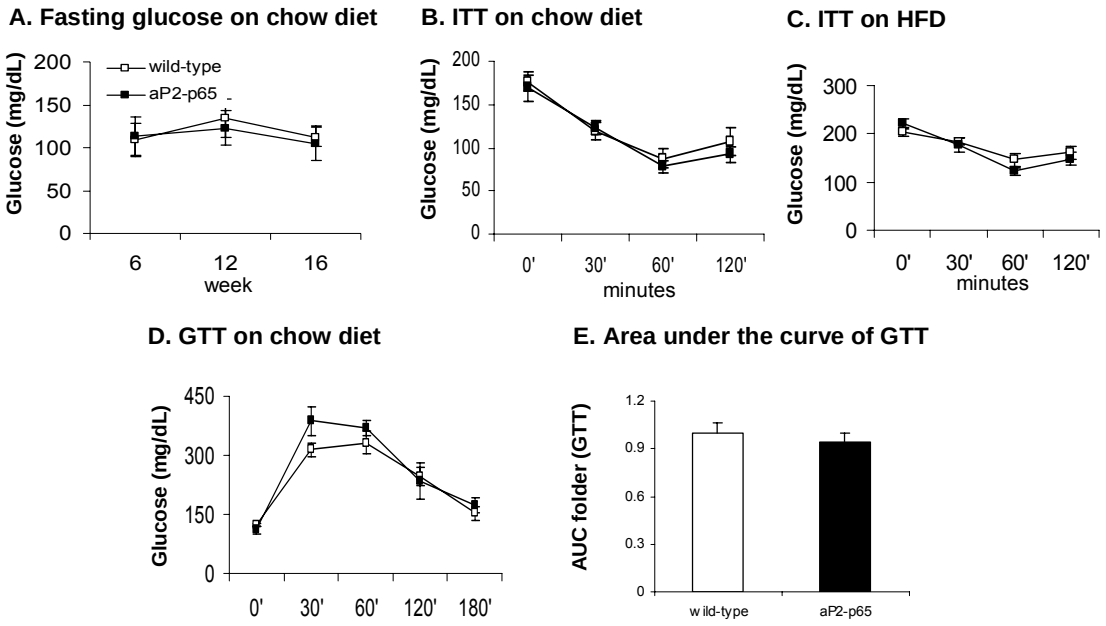

Figure 5. Insulin sensitivity and glucose response in aP2-p65 mice. (A) Fasting glucose. Tail vein blood was used for glucose assay after 12 hr fasting during the period of chow diet feeding; (B) ITT on chow diet. ITT was done at 14 weeks of age after 4 hr fasting; (C) ITT on HFD. ITT was done at 6 weeks on HFD (at 22 weeks of age); (D) GTT on chow diet. GTT was done at 16 weeks of age; (E) Area under the curve fold calculated from GTT result. For A-C, n=10, for D-E, n=6 in WT or Tg group. Values are the mean ± SE. For *P<0.05, **P<0.001 by Student's t test.

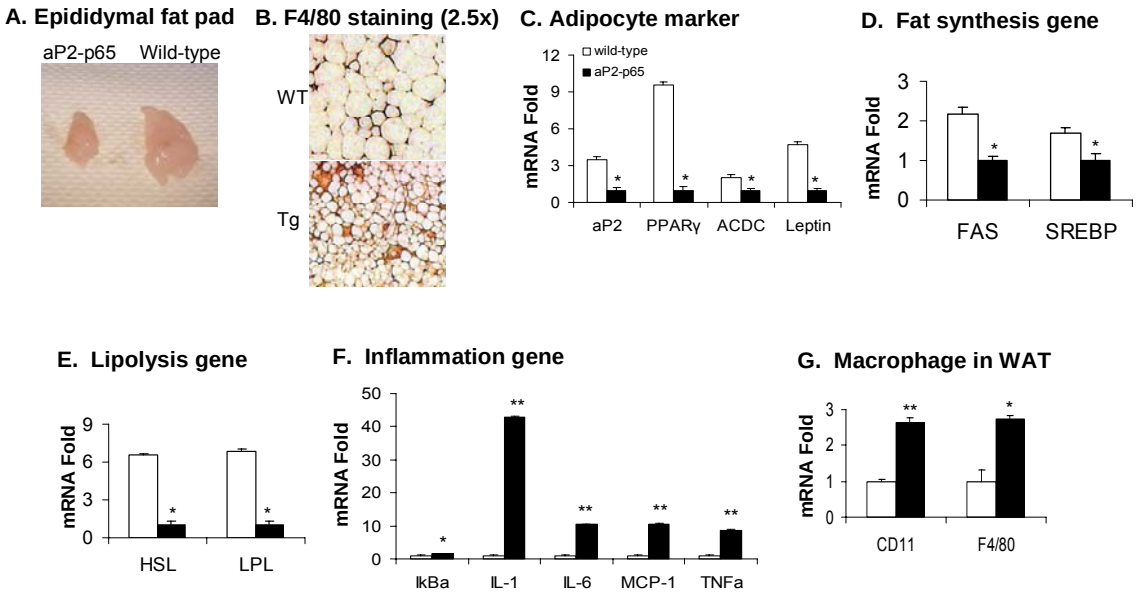

Figure 6. Decreased adipose tissue function as indicated by gene expression. (A) Intact epididymal fat pad was collected at the age of 20 weeks and picture was taken to compare the fat pad size of the two type of mouse; (B) F4/80 immunostaining in WAT; (C, D ,E, F and G) Epididymal fat tissue was collected at the age of 20 weeks and RNA was extracted; (C) Adipocyte genes. Relative mRNA folders of aP2, PPARγ, adiponectin (ACDC) and leptin are determined by real time RT-PCR; (D) Fat synthesis genes. Relative mRNA folders of FAS and SREBP are determined by real time RT-PCR; (E) Lipolysis genes. Relative mRNA folders of HSL and LPL are determined by real time RT-PCR; (F) Inflammation genes. Relative mRNA folders of IκBa, IL1, IL6, TNF alpha and MCP1 are determined by real time RT-PCR; (G) Macrophage marker, CD11 and F4/80 expression in WAT, determined by real time RT-PCR. For C-G, n=6 in WT or Tg group. Values are the mean ± SE. *P<0.05, **P<0.001 by Student's t test.

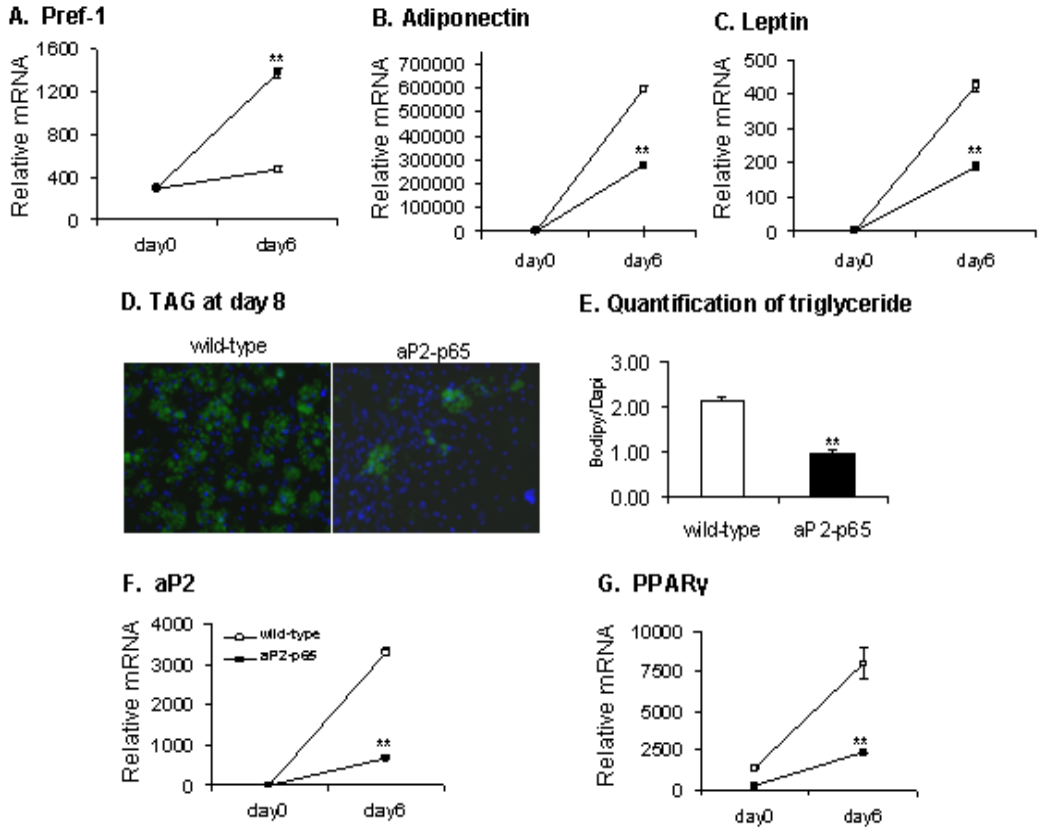

Figure 7. Adipogenesis Study in vitro**.** Inguinal fat tissue was collected from both aP2-p65 transgenic mice and wild-type mice (at the age of 5 weeks). After digested with collagenase, the preadipocytes were isolated and cultured in vitro. When the cells reach confluence, adipogenic induction medium was added to induce differentiation. At the induction day 0, 2, 3, 6 and 8, pictures were taken and RNA was taken from part of cells. (A, B and C). Change of adipocyte marker genes mRNA level through adipogenesis. (A) Pref-1 relative mRNA level through adipogenesis; (B) Adiponectin relative mRNA level through adipogenesis; (C) Leptin relative mRNA level through adipogenesis; (F) aP2 relative mRNA level through adipogenesis; (G) PPARγ relative mRNA level through adipogenesis; (D) Fluorescence images taken on the 8 day of differentiation of the preadipocytes, demonstrating the adipogenesis of WT mouse and Tg mice; (E) Guantification of triglyceride in adipocytes. BODIPY and DAPI staining was used to illustrate the TG amount in the mature adipocytes. Green areas demonstrate the triglyceride droplets, blue areas demonstrate nucleus. BODIPY/ DAPI means the triglyceride amount adjusted by cell amount. For A, B, C, F and G, n=3 in WT or Tg group. For D and E, n=6 pictures of cells were taken in wild-type or aP2-p65 group to calculate the average of the quantification. Values are the mean ± SE. *P<0.05, **P<0.001 by Student's t test.

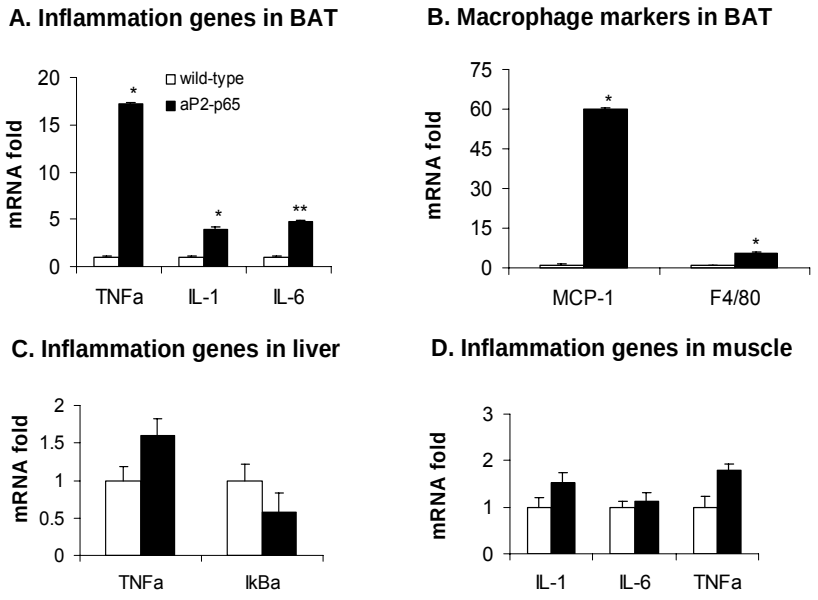

Figure 8. Inflammation status in Brown Adipose Tissue and Other Tissues. Brown adipose tissue, liver and skeletal muscle was collected at the age of 20 weeks and RNA was extracted. (A) Inflammation gene expression in BAT. Relative mRNA folder of TNF-α, IL-1 and IL-6 is determined by real time RT-PCR; (B) Relative mRNA folder of macrophage markers F4/80, MCP1 in BAT, determined by real time RT-PCR; (C) Relative mRNA folder of inflammation gene TNF-α and IκBα in liver, other inflammation genes were not detected in real time RT-PCR. (D) Relative mRNA folder of inflammation gene IL-1, IL-6 and TNF-α in liver. For A-D, n=6 in WT or Tg group. Values are the mean ± SE. *P<0.05, **P<0.001 by Student's t test.

Similar to our Tg mice, the aP2-Lepr-b mice had less adipogenic capacity but more severe insulin resistance than db/db and WT mice when fed a high-fat diet[65]. Morphologic examination showed that ectopic lipid deposition happened widely in heart tissue, liver and other tissues of the aP2-Lepr-b mice. The findings suggest that the function of the adipocyte to store surplus calories during overnutrition has a great effect on systemic insulin resistance. Thus we might hypothesize that the impaired adipogenesis in adipose tissue of Tg mice also induces ectopic lipid deposition in other tissues, leading to the systemic insulin resistance. Further study may switch to other organs and tissues including liver and skeletal muscle, which may take more important role in systemic metabolism.

P65 and Inflammation: In this study, we demonstrated that overexpression of NF-κB P65 caused chronic inflammation in adipose tissue. This is supported by an increase in macrophage infiltration, type 1 macrophages, and expression of inflammatory cytokines (TNF-α, IL-1, IL-6, and MCP-1). The inflammation may be a result of elevated expression of p65 in macrophages since p65 mRNA is increased in the macrophages of Tg mice. The p65 expression enhances macrophage expression of inflammatory cytokines in the basal condition, and as well in LPS-stimulated condition. The inflammation is expected since NF-κB p65 is a major transcriptional activator for inflammatory genes.

The expression of p65 in adipocytes may also contribute to the inflammation. However, the direct effect may be limited since the inflammatory activity of adipocytes is much lower than that of macrophages [66]. It is possible that adipocytes contribute to the inflammation by secretion of MCP-1 and IL-6, which promote macrophage infiltration and activation.

CHAPTER 3

SUMMARY AND CONCLUSION

In this study, we made the fat-specific transgenic mice for NF-κB p65. The p65 expression was observed in adipocytes and in macrophages. This expression pattern leads to a lean phenotype in the Tg mice with elevated energy expenditure and a defect in adipogenesis. This lean phenotype prevents adulthood obesity and dietary obesity. However, it does not provide benefit to the protection of systemic insulin sensitivity. The underlying mechanism might be ectopic fat deposition in the Tg mice. There is no difference between the Tg and WT mice in insulin sensitivity on Chow (low fat) or HFD. The p65 gene modification seems to have no influence on reproductivity, embryo development and postnatal growth in the Tg mice.

REFERENCES

1. Ogden, C.L., et al., *Prevalence of overweight and obesity in the United States, 1999-2004.* Jama, 2006. **295**(13): p. 1549-55.

2. Finkelstein, E.A., I.C. Fiebelkorn, and G. Wang, *National medical spending attributable to overweight and obesity: how much, and who's paying?* Health Aff (Millwood), 2003. **Suppl Web Exclusives**: p. W3-219-26.

3. Kissebah, A.H., *Insulin resistance in visceral obesity.* Int J Obes, 1991. **15 Suppl 2**: p. 109-15.

4. Klein, S., *The case of visceral fat: argument for the defense.* J Clin Invest, 2004. **113**(11): p. 1530-2.

5. Brake, D.K. and C.W. Smith, Flow cytometry on the stromal-vascular fraction of white adipose tissue. Methods Mol Biol, 2008. **456**: p. 221-9.

6. Wellen, K.E. and G.S. Hotamisligil, *Obesity-induced inflammatory changes in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1785-8.

7. Coppack, S.W., *Pro-inflammatory cytokines and adipose tissue.* Proc Nutr Soc, 2001. **60**(3): p. 349-56.

8. Harkins, J.M., et al., Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. J Nutr, 2004. **134**(10): p. 2673-7.

9. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.

10. Di Gregorio, G.B., et al., Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. Diabetes, 2005. **54**(8): p. 2305-13.

11. Loffreda, S., et al., *Leptin regulates proinflammatory immune responses.* Faseb J, 1998. **12**(1): p. 57-65.

12. Whitehead, J.P., et al., *Adiponectin--a key adipokine in the metabolic syndrome.* Diabetes Obes Metab, 2006. **8**(3): p. 264-80.

13. Steppan, C.M., et al., *Activation of SOCS-3 by resistin.* Mol Cell Biol, 2005. **25**(4): p. 1569-75.

14. Truong, M.T., et al., Focal FDG uptake in mediastinal brown fat mimicking malignancy: a potential pitfall resolved on PET/CT. AJR Am J Roentgenol, 2004. **183**(4): p. 1127-32.

15. Truong, M.T., R.F. Munden, and B. Movsas, *Imaging to optimally stage lung cancer: conventional modalities and PET/CT.* J Am Coll Radiol, 2004. **1**(12): p. 957-64.

16. Cinti, S., et al., Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res, 2005. **46**(11): p. 2347-55.

17. Cancello, R., et al., Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes, 2005. **54**(8): p. 2277-86.

18. Boden, G., *Obesity and free Fatty acids.* Endocrinol Metab Clin North Am, 2008. **37**(3): p. 635-46.

19. Delarue, J. and C. Magnan, *Free fatty acids and insulin resistance.* Curr Opin Clin Nutr Metab Care, 2007. **10**(2): p. 142-8.

20. Tsiotra, P.C. and C. Tsigos, *Stress, the endoplasmic reticulum, and insulin resistance.* Ann N Y Acad Sci, 2006. **1083**: p. 63-76.

21. Ye, J., Role of insulin in the pathogenesis of free fatty acid-induced insulin resistance in skeletal muscle. Endocr Metab Immune Disord Drug Targets, 2007. **7**(1): p. 65-74.

22. Heilbronn, L.K. and L.V. Campbell, Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr Pharm Des, 2008. **14**(12): p. 1225-30.

23. Valerio, A., et al., TNF-alpha downregulates eNOS expression and mitochondrial biogenesis in fat and muscle of obese rodents. J Clin Invest, 2006. **116**(10): p. 2791-8.

24. Valladares, A., et al., TNF-alpha inhibits UCP-1 expression in brown adipocytes via ERKs. Opposite effect of p38MAPK. FEBS Lett, 2001. **493**(1): p. 6-11.

25. Hadri, K.E., et al., Differential regulation by tumor necrosis factor-alpha of beta1-, beta2-, and beta3-adrenoreceptor gene expression in 3T3-F442A adipocytes. J Biol Chem, 1997. **272**(39): p. 24514-21.

26. Nisoli, E., et al., Tumor necrosis factor-alpha induces apoptosis in rat brown adipocytes. Cell Death Differ, 1997. **4**(8): p. 771-8.

27. Nisoli, E., et al., Tumor necrosis factor alpha mediates apoptosis of brown adipocytes and defective brown adipocyte function in obesity. Proc Natl Acad Sci U S A, 2000. **97**(14): p. 8033-8.

28. Masaki, T., et al., Tumor necrosis factor-alpha regulates in vivo expression of the rat UCP family differentially. Biochim Biophys Acta, 1999. **1436**(3): p. 585-92.

29. Plata-Salaman, C.R., Central nervous system mechanisms contributing to the cachexia-anorexia syndrome. Nutrition, 2000. **16**(10): p. 1009-12.

30. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science, 1993. **259**(5091): p. 87-91.

31. Uysal, K.T., et al., Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature, 1997. **389**(6651): p. 610-4.

32. Stephens, J.M. and P.H. Pekala, Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. J Biol Chem, 1991. **266**(32): p. 21839-45.

33. Kawakami, M., et al., Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in 3T3-L1 cells. J Biochem, 1987. **101**(2): p. 331-8.

34. Kelley, D.S., Modulation of human immune and inflammatory responses by dietary fatty acids. Nutrition, 2001. **17**(7-8): p. 669-73.

35. Zhang, H.H., et al., Tumor necrosis factor-alpha stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. Diabetes, 2002. **51**(10): p. 2929-35.

36. Souza, S.C., et al., TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes. J Cell Biochem, 2003. **89**(6): p. 1077-86.

37. Ryden, M., et al., *Targets for TNF-alpha-induced lipolysis in human adipocytes.* Biochem Biophys Res Commun, 2004. **318**(1): p. 168-75.

38. Tamori, Y., et al., Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes. Diabetes, 2002. **51**(7): p. 2045-55.

39. Sakurai, T., et al., Exercise training enhances tumor necrosis factor-alpha-induced expressions of anti-apoptotic genes without alterations in caspase-3 activity in rat epididymal adipocytes. Jpn J Physiol, 2005. **55**(3): p. 181-9.

40. Ohsumi, J., et al., Troglitazone prevents the inhibitory effects of inflammatory cytokines on insulin-induced adipocyte differentiation in 3T3-L1 cells. Endocrinology, 1994. **135**(5): p. 2279-82.

41. Ruan, H., et al., Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. Diabetes, 2002. **51**(11): p. 3176-88.

42. Cawthorn, W.P., et al., Tumour necrosis factor-alpha inhibits adipogenesis via a beta-catenin/TCF4(TCF7L2)-dependent pathway. Cell Death Differ, 2007. **14**(7): p. 1361-73.

43. Miles, P.D., et al., Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency. J Clin Invest, 2000. **105**(3): p. 287-92.

44. Peraldi, P., M. Xu, and B.M. Spiegelman, Thiazolidinediones block tumor necrosis factor-alpha-induced inhibition of insulin signaling. J Clin Invest, 1997. **100**(7): p. 1863-9.

45. Ruan, H., H.J. Pownall, and H.F. Lodish, Troglitazone antagonizes tumor necrosis factor-alpha-induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-kappaB. J Biol Chem, 2003. **278**(30): p. 28181-92.

46. Cai, D., et al., Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med, 2005. **11**(2): p. 183-90.

47. Lappas, M., et al., Sulfasalazine and BAY 11-7082 interfere with the nuclear factor-kappa B and I kappa B kinase pathway to regulate the release of proinflammatory cytokines from human adipose tissue and skeletal muscle in vitro. Endocrinology, 2005. **146**(3): p. 1491-7.

48. Nai, Y.J., et al., Prevention of cancer cachexia by pyrrolidine dithiocarbamate (PDTC) in colon 26 tumor-bearing mice. JPEN J Parenter Enteral Nutr, 2007. **31**(1): p. 18-25.

49. Zhou, W., et al., *Role of NF-kappaB and cytokine in experimental cancer cachexia.* World J Gastroenterol, 2003. **9**(7): p. 1567-70.

50. Kuroda, K., et al., Interleukin 6 is associated with cachexia in patients with prostate cancer. Urology, 2007. **69**(1): p. 113-7.

51. Gao, Z., et al., Regulation of nuclear translocation of HDAC3 by IkappaBalpha is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. J Biol Chem, 2006. **281**(7): p. 4540-7.

52. Lee, J.Y., et al., Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem, 2003. **278**(39): p. 37041-51.

53. Gao, Z., et al., Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes. Mol Endocrinol, 2004. **18**(8): p. 2024-34.

54. Gao, Z., et al., Coactivators and corepressors of NF-kappaB in IkappaB alpha gene promoter. J Biol Chem, 2005. **280**(22): p. 21091-8.

55. Zhong, H., R.E. Voll, and S. Ghosh, Phosphorylation of NF-kappa B p65 by PKA

stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell, 1998. **1**(5): p. 661-71.

56. Beg, A.A., et al., Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature, 1995. **376**(6536): p. 167-70.

57. Neurath, M.F., et al., Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. Nat Med, 1996. **2**(9): p. 998-1004.

58. Suzawa, M., et al., Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. Nat Cell Biol, 2003. **5**(3): p. 224-30.

59. Ye, J., et al., Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. Am J Physiol Endocrinol Metab, 2007. **293**(4): p. E1118-28.

60. Shoelson, S.E., J. Lee, and A.B. Goldfine, *Inflammation and insulin resistance.* J Clin Invest, 2006. **116**(7): p. 1793-801.

61. Simon, M.F., et al., Lysophosphatidic acid inhibits adipocyte differentiation via lysophosphatidic acid 1 receptor-dependent down-regulation of peroxisome proliferator-activated receptor gamma2. J Biol Chem, 2005. **280**(15): p. 14656-62.

62. Jones, J.R., et al., Deletion of PPARgamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. Proc Natl Acad Sci U S A, 2005. **102**(17): p. 6207-12.

63. Tseng, Y.H., et al., Prediction of preadipocyte differentiation by gene expression reveals role of insulin receptor substrates and necdin. Nat Cell Biol, 2005. **7**(6): p. 601-11.

64. Kamei, N., et al., Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. J Biol Chem, 2006. **281**(36): p. 26602-14.

65. Wang, M.Y., et al., Adipogenic capacity and the susceptibility to type 2 diabetes and metabolic syndrome. Proc Natl Acad Sci U S A, 2008. **105**(16): p. 6139-44.

66. Fain, J.N., S.W. Bahouth, and A.K. Madan, *TNFalpha release by the nonfat cells of human adipose tissue.* Int J Obes Relat Metab Disord, 2004. **28**(4): p. 616-22.

VITA

Tianyi Tang was born in March, 1982, as the single child to parents Yaoming Tang and Meibin Li in a city of Hunan province, People's Republic China. In her teenage, she moved to Guangzhou City in Guangdong Province with her family. In 1998, Tianyi was enrolled into the medical school of Sun Yat-sen University after graduated from high school and was awarded with bachelor's degree in medicine in 2003. At the same year, Tianyi got the admission of the graduate school of Sun Yat-sen University and then majored in internal medicine from 2003 to 2006. In the spring of 2007, Tianyi entered a graduate program in the School of Human Ecology in Louisiana State University and worked as a research assistant in Pennington Biomedical Research Center under the direction of Dr. Jianping Ye.

During the graduate study in Sun Yat-sen University, Tianyi was trained as junior clinical doctor in internal medicine in the first affiliated hospital of the university. Half of her graduate study was concentrating on endocrinology. Her thesis was about the glucose metabolism of a case of rare genetic insulin resistance syndrome. As one of the major researcher in this project, Tianyi was invited to the 7th Symposium on Molecular Diabetology in Asia (2005) in Hong Kong. After she came to the United States and switched her interest into nutrition, she has been investigating the association between inflammation and insulin resistance for nearly two years. Tianyi now is working hard on her new paper and her thesis for the master's degree in nutrition. After completing her current program, Tianyi will continue to do research in nutrition and contribute to the everlasting resources for the scientific community.