The Genetic Ablation of TNF-α Attenuates Wnt-Signaling and Adiposity in High Fat Diet-Induced Obese Mice

Jinchao Li1, Susan Kim2, Seok-Yeong Yu1, Ying Tang1, Young-Cheul Kim1,3, Soonkyu Chung1 and Zhenhua Liu1,2,*

1Department of Nutrition, School of Public Health and Health Sciences, University of Massachusetts, USA
2Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, USA
3Molecular and Cellular Biology Graduate Program, University of Massachusetts, USA

Abstract
A chronic low-grade inflammation is considered as a consequence of obesity, and linked with multiple complications. However, it is under-investigated how inflammatory cytokines mediate adipogenesis. This study investigated the role of Tumor Necrosis Factor α (TNF-α) on adipogenesis is over high-fat diet feeding. Three groups of wild type or TNF-α-/- mice with the same C57BL/6 genetic background were utilized in this study: wild type fed with a low-fat diet (WT-LFD), wild type fed with a high-fat diet (WT-HFD), and TNF-α-/- fed with a HFD (TNF-HFD). After 16-wk feeding, inflammatory cytokine, Wnt pathway and adipogenesis-related genes were analyzed. HFD feeding increased body weight in both WT-HFD and TNF-HFD groups, but genetic ablation of TNF-α attenuated HFD-induced obesity. In visceral adipose tissues, HFD elevated Wnt/β-catenin signaling, indicated by decreased phospho-GSK3β and active β-catenin, two key components within the Wnt pathway, and dysregulated adipogenesis, indicated by reduced PPARγ/CEBPα expressions. Whereas, the deletion of TNF-α suppressed Wnt-signaling, and restored expressions of adipogenesis-related genes, which were otherwise decreased in the HFD-induced obese animals. These findings demonstrated a critical role of TNF-α in the regulation of Wnt-signaling and adiposity in mice over a HFD feeding, indicating HFD-induced adipocyte dysfunction could be mitigated by targeting TNF-α and Wnt-signaling.

Keywords
Obesity, Inflammation, TNF-α, Wnt pathway, Adipogenesis, PPARγ

Introduction
It is well-established that obesity, with inflammation as one of the critical mechanisms, linked to a range of chronic diseases such as type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD), cardiovascular disease (CVD), and cancer [1]. Obesity is well-accepted as a chronic inflammatory condition as a consequence of hypertrophic adipocyte expansion [2]. Whereas, how inflammatory cytokines reverse mediates adipogenesis is less investigated. To understand this mechanism and prevent chronic over nutrition-induced adipocyte dysfunction represents a promising approach to diminish the epidemic of obesity and its associated medical complications in our society [3,4].

Adipose tissue, as an endocrine organ, plays an important role in maintaining the whole-body energy metabolism [5]. In order to maintain its function, a constant differentiation of adipocytes (adipogenesis) is necessary in adipose tissue [6]. During the adipogenesis from undifferentiated pre-adipocyte to lipid-loaded mature adipocytes, Wnt/β-catenin signaling participates in the transition and aberrant over-activation of Wnt-signaling results in the interruption of this process by inhibiting PPARγ expression [7,8]. Among many transcriptional factors, PPARγ is one of the major transcriptional factors to orchestrate the adipogenesis. PPARγ regulates lipid and glucose metabolism, and PPARγ target genes critically involve in lipid uptake and adipogenesis [9]. PPARγ agonists have been used in the treatment of hyperlipidemia and hyperglycemia [10], and it has been implicated in the pathology of numerous diseases including cardiovascular diseases and cancer [11]. Therefore, it is important to understand the regulation of PPARγ expression in obesity, thereby providing feasible av-
enues to properly manage adipogenesis and related complications.

Tumor necrosis factor-alpha (TNF-α), which is one of pro-inflammatory cytokines found to be increased in obesity, has been considered an important mediator that causes metabolic dysregulations in the body via adipose tissue dysfunction [12]. Previous animal studies with TNF-α knockout (TNF-α−/−) mice showed that TNF-α deficiency attenuated insulin resistance in peripheral tissues and prevented excessive hepatic lipid accumulation in diet-induced obese mice [13-16]. However, the cellular mechanism(s), by which TNF-α mediates adipose dysfunction has not been clearly defined. This study aimed to investigate the role of TNF-α in the regulation of Wnt/β-catenin signaling in adipogenesis in mice over HFD feeding. We hypothesized that obesity-promoted TNF-α activates Wnt/β-catenin signaling in adipose tissue, and that its constant activation inhibits the production of PPARγ, thereby leading to further adipocyte dysfunction.

Methods

Animals and experimental diets

TNF-α−/− mice were purchased from Jackson laboratory (Bar Harbor, ME, USA). It was originally created on 129S/6 background, but has been backcrossed to C57BL/6 for >10 generations. Mice were housed under conditions of controlled temperature (22 °C - 25 °C) and illumination (12:12 hour light-dark cycle, lights on between 7:00 and 19:00) with food and water provided ad libitum. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst.

All the C57BL/6 wild type and TNF-α−/− mice were initially fed a standard rodent chow diet (Teklad®8604, Envigo, Madison, WI). At 8 weeks of age, animals were divided into 3 groups (26 animals (13 males and 13 females) /group): wild type fed with a low-fat diet (WT-LFD, 10 % of total calorie from fat), wild type fed with a high-fat diet (WT-HFD, 60 % of total calorie from fat), and TNF-α−/− fed with the same high-fat diet (TNF-α−/−-HFD). A high-fat diet, which is commonly used to induce obesity, was purchased from Research Diets (HFD: D12492 vs. LFD: D12450B, New Brunswick, NJ). The dietary compositions were shown in Table 1. As significant gender differences in body weight gain and metabolic parameters exist with male mice more sensitive to high fat induced-induced obesity [17,18], in addition to the body weight and body composition, only male animals were used for molecular analysis in this study to avoid the confounding from gender. At the end of the experiment, mice were euthanized by CO2 asphyxiation followed by cervical dislocation. Exsanguination was performed by cardiac puncture and blood was collected. The abdomen was opened and epididymis fats were collected into foil packets and frozen in liquid N2 for subsequent RNA and protein assays.

Inflammatory cytokine assays

The inflammatory cytokines were measured by a chemiluminescence assay using the QuickPlex SQ 120 (Meso Scale Diagnostics, Rockville, MD). Assays were performed according to the manufacturer’s instructions. Briefly, the protein samples including inflammatory cytokines were isolated from visceral fat tissues with lysis buffer prepared according to the protocol for the electrochemiluminescence assay (Meso Scale Discovery, Rockville, MD). Then 25 µl of calibrator standards or samples were added to each well of a 96-well plate, on the bottom of which antibodies for 3 cytokines (IFN-γ, IL-1β, and IL-6) were coated. After washing for 3 times, 50 µl of the detection antibody solution was added to each well. A four-parameter logistic fit curve was generated for each analyte using the standards and the levels of inflammatory cytokines in samples were calculated accordingly. Cytokines are express as ng of cytokine per mg protein. All standards and samples were measured in duplicate.

Real-time PCR for gene expression

Total RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA). The concentration as well as purity of RNA samples was determined by NanoDrop 2000 (Thermo Scientific, Waltham, MA). The first-strand cDNAs were synthesized from 0.5 µg total RNAs using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR was performed on the ViiA 7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the SYBR green PCR reagent kit (Invitrogen, Carlsbad, CA) with the following thermal cycling condi-

| Table 1: Dietary composition for the high fat diet (60 kcal%) and low fat control diet (10 kcal%)*. |
|-----------------------------------------------|
| Ingredient (g/kg) | Low fat diet | High fat diet |
|-------------------|-------------|--------------|
| Casein            | 189.6       | 258.5        |
| L-Cystine         | 2.8         | 3.9          |
| Corn Starch       | 298.6       | 0            |
| Maltodextrin      | 33.2        | 161.5        |
| Sucrose           | 331.8       | 88.9         |
| Cellulose         | 47.4        | 64.6         |
| Soybean Oil       | 23.7        | 32.3         |
| Lard              | 19          | 316.6        |
| Mineral Mix S10026| 9.5         | 12.9         |
| DiCalcium Phosphate| 12.3      | 16.8         |
| Calcium Carbonate | 5.2         | 7.1          |
| Potassium Citrate | 15.6        | 21.3         |
| Vitamin Mix V10001| 9.5         | 12.9         |
| Choline Bitartrate| 1.9         | 2.6          |
| Total             | 1000        | 1000         |
| **Energy(%kcal)** | **Low Fat Diet** | **High Fat Diet** |
| Protein           | 20          | 20           |
| Carbohydrate      | 70          | 20           |
| Fat               | 10          | 60           |
| Total             | 100         | 100          |

*Diets: The low fat diet (D12450B) and High fat diet (D12492) were made by Research Diets, Inc.
The Wnt-signaling pathway

At the beginning of the experiment (8 wks of age), there were no differences across the 3 groups in terms of body weight. As expected, after 16 weeks of high fat diet feeding, the body weight of both wild type mice (WT-HFD) and TNF-α/- (TNF-HFD) significantly increased when compared with that of wild type mice fed a low fat diet (WT-LFD). It is noteworthy that the genetic deletion of TNF-α reduced the sensitivity of high fat diet-induced obesity. Only after 7 weeks, the body weight of the wild type animals fed a high fat diet (WT-HFD) increased to a degree that was significantly higher than the body weight of those animals fed a low fat diet (WT-LFD), whereas a statistical difference was not observed for the TNF-α/-animals (TNF-HFD) until 13 weeks of a high fat diet feeding (p < 0.05). The body weight of the WT-HFD group were significantly higher than that of the TNF-HFD group after feeding for the high fat diet for 9 weeks (p < 0.05). By the end of the experiment, the body weight gains were 53.7% for the WT-LFD group, and 88.9% for the TNF-HFD group. Compared to the WT-LFD group, the body weights were 38.5% higher for the WT-HFD group, but only 15.5% for the TNF-HFD group (p < 0.05) (Figure 1a).

The body composition data, measured by Magnetic Resonance Imaging (MRI), showed that, comparing to the WT-LFD group, the fat mass in the WT-HFD increased by 19.98% (16.35 ± 0.86% vs. 36.33 ± 1.82%, p < 0.01), but it only increased by 14.35% (30.70 ± 1.53% for the TNF-HFD, p < 0.01). It is also significantly different between the WT-HFD and TNF-HFD group (p < 0.05). On the contrast, the lean mass in the WT-HFD decreased by 16.48% (72.45 ± 0.78% vs. 55.97 ± 1.68%, p < 0.01), but it only decreased by 11.97% (60.48 ± 1.28% for the TNF-HFD, p < 0.01). It is also significantly different between the WT-HFD and TNF-HFD group (p < 0.05) (Figure 1b).

Inflammatory Cytokines

After observing the differentiated body weight change among groups, we measured 3 inflammatory cytokines in the visceral adipose tissue (IFNγ, IL-1β and IL-6). When compared to the WT-LFD group, the high fat diet (WT-HFD) significantly increased protein levels of IFNγ by 120.3% (p < 0.01), IL-1β by 29.5% (p < 0.05) and IL-6 by 100.6% (p < 0.001). However, the genetic ablation of TNF-α diminished the these elevations and the levels of IFNγ and IL-6 in the TNF-HFD group were significantly lower than in the WT-HFD groups (p < 0.05) with a numerical decrease of IL-1β (p = 0.07) (Figure 2).

The Wnt-signaling pathway

To investigate the Wnt-signaling pathway, we examined
Figure 1: The body weight and composition of animals fed a high fat (60 kcal% fat) or a low fat (10 kcal% fat) diet. a) Growth curve during the 16 weeks of experiment from the age of 8 weeks to 24 weeks; b) Body composition measured by Magnetic Resonance Imaging (MRI); c) Representative mouse pictures from each group. WT-LED: Wild type fed with a low-fat diet (10 kcal% fat), WT-HFD: Wild type fed with a high-fat diet (WT-HFD, 60 kcal% fat), and TNF-HFD: TNF-α⁻/⁻ fed with a high-fat diet (WT-HFD, 60 kcal% fat). * represents a significant difference from the WT-LFD group. # represents a significant difference between the WT-HFD and the TNF-HFD groups. n = 26 animals/group.

Figure 2: The levels of inflammatory cytokines in visceral adipose tissue. WT-LED: Wild type fed with a low-fat diet (10 kcal% fat), WT-HFD: Wild type fed with a high-fat diet (WT-HFD, 60 kcal% fat), and TNF-HFD: TNF-α⁻/⁻ fed with a high-fat diet (WT-HFD, 60 kcal% fat). n = 7 male animals/group.
the protein levels of phosphorylated GSK3β and active β-catenin as well as the transcriptional levels of Wnt-signaling downstream target genes, C-Myc and Cyclin D1. As shown in figure 3a and figure 3b, when compared to WT-LFD, high fat diet (WT-HFD) significantly increased the protein levels for both phospho-GSK3β at the Ser 9 site (the inactive form) and active β-catenin (p < 0.01), whereas the genetic deletion of TNF-α (TNF-HFD) significantly diminished these effects (p < 0.01). After observing the mitigation of phosphorylated GSK3β and active β-catenin by TNF-α, we further examined the transcriptional expression of Wnt-signaling downstream genes. As shown in figure 3c, when compared to the WT-HFD group, the deletion of TNF-α (TNF-HFD group) reduced the expression of C-Myc and Cyclin D1 (p < 0.01) which were otherwise up regulated by high fat diet (p < 0.001, WT-LFD vs. WT-HFD).

**Adipogenesis**

To investigate the influence of TNF-α on adipocyte dysfunction in high fat-induced obesity, we analyzed the expression, at both transcriptional and protein level, of PPARγ and CEBPα that are two critical transcriptional factors involved in adipogenesis. We found that, after a 16-wk high fat diet feeding, the transcriptional expression of Ppary and Cebpα were significantly suppressed (p < 0.001) by high fat diet (WT-HFD vs WT-LFD), whereas the genetic deletion of TNF-α attenuated this effect (TNF-HFD vs WT-HFD, p < 0.05) (Figure 4). The analysis of the protein levels of these two adipogenesis-related genes recapitulated the transcriptional data (Figure 4).

**Discussion**

Obesity with adipocyte hypertrophic expansion represents a low-grade inflammatory condition [14,19-21], and the increased inflammatory cytokines are generally considered as a consequence of obesity. This study, on the contrast, demonstrated that genetic ablation of TNF-α attenuated HFD-induced obesity, potentially via attenuating Wnt/β-catenin signaling and restoring the adipogenic PPARγ/CEBPα expression, suggesting that TNF-α plays a causal role in adipocyte dysfunction during adipocyte hypertrophic expansion.

Since the initial discovery of escalated expression of TNF-α in adipose tissue by Hotamisligil and colleagues in 1993 [14], it is well-accepted that the adipose tissue is an active endocrine organ, which releases >50 different cytokines and adipokines [19,20]. In addition to the origins of inflammatory cytokine from adipocytes, adipocyte hypertrophy leads to the infiltration of immune cells, especially macrophages, and these immune cells significantly contribute to the production of pro-inflammatory cytokines [22]. Whereas, it is less understood how inflammatory cytokines influence adiposity, though a few pro-inflammatory cytokines have been shown possessing important metabolic effects, including effect on lipid metabolism [23,24].

Studies pertaining the role of TNF-α on adipocyte dysfunction and obesity are limited and results are highly inconsistent. The study performed by Uysal, et al. in 1997 [16] showed that the body weights were similar between the wild type and TNF-α−/− animals regardless feeding with a LF or HF diet throughout the 16-week study, but a significantly decreased body weight for the TNF-α−/− model fed a HF diet were observed in a study from others [13]. On the contrast, two other studies in 2012 [15] and 2016 [25] showed that TNF-α−/− mice were even prone to gain weight on HF diet when...
Figure 4: Genetic ablation of TNF-α attenuated the high fat diet-reduced mRNA and protein levels of adipogenesis-related genes. a) Transcriptional expression of adipogenesis-related gene Pparγ and Cebpα. c) Western blotting of PPARγ and CEBPα; b) The density of the western blotting band analyzed by Image J. The ratio are the density of the proteins vs the density of the control GAPDH band based on the Image J program. WT-LFD: Wild type fed with a low-fat diet (10 kcal% fat), WT-HFD: Wild type fed with a high-fat diet (WT-HFD, 60 kcal% fat), and TNF-HFD: TNF-α−/− fed with a high-fat diet (WT-HFD, 60 kcal% fat). Values are means ± SEMs, n = 4 animals/group. * represents a p < 0.05, ** represents a p < 0.01, *** represents a p < 0.001.

Figure 5: PPARγ and Wnt/β-catenin signaling in the regulation of adipogenesis. At the beginning of high-fat diet feeding, our body may respond via stimulating the progression from preadipocyte to matured adipocyte, resulting in hyperplasia characterized by increased number of matured adipocytes. These adipocytes may secrete inflammatory factors, leading to increased level of PPARγ and the proliferation of adipocytes. Whereas, with the continuous high-fat diet feeding, the over-uptake of lipids lead the adipocyte to the hypertrophic stage, which results to inflammation in the adipose tissue. The increased inflammatory cytokines may activate Wnt-signaling, which further inhibits PPARγ expression and leads to the dysregulation of adipogenesis.
compared wild type animals. The study of Uysal, et al. [16] also showed that the genetic absence of TNF-α receptors did not attenuate body weight in genetically-induced obese animals (ob/ob) when compared to those ob/ob mice without the deletion of TNF-α receptors. However, another study [26] showed that the ablation of TNF-α receptor protected against diet-induced obesity. These inconsistent results require further investigation to understand the cellular action of TNF-α on adipogenesis.

Diet-induced adiposity includes two stages: hyperplasia characterized by increased number of adipocytes, and hypertrophy featured with enlarged adipocytes. For a short-term over nutrition, our body may respond via stimulating the progression from pre-adipocyte to matured adipocyte, resulting in increased number of adipocytes called adipose hyperplasia. It is reported that, as early as 2 wks of HF diet feeding, an increased expression of PPARγ was observed in the liver tissue [27]. Whereas in chronic over nutrition, the over-uptake of lipids lead the adipocyte to the hypertrophic stage, which results in inflammation in the adipose tissue. Therefore, inflammatory cytokines may play regulatory roles on adipogenesis primarily in the latter hypertrophic stage when inflammation occurs (Figure 5). In our current study, animals were fed with a HFD (60 kcal% fat) for a long period (16 wks), and had reached the hypertrophic phase as indicated by increased inflammation. Our data clearly demonstrated that the genetic ablation of TNF-α attenuated Wnt/β-catenin signaling, restored the expression of PPARγ, and thereby suppressed HFD-induced obesity over a long-term HFD feeding. Our results may offer an explanation for the previous inconsistent results. TNF-α plays a regulatory role on adipogenesis primarily at the latter hypertrophic stage when the inflammation occurs in the adipose tissue. In those previous studies, due to the either low fat content in the diet or the duration of feeding, animals in some of those studies might not reach the hypertrophic stage, and thereby resulted in inconsistent results.

Adipogenesis can be divided into two stages: Mesenchymal stem cells (MSCs) “commitment” to pre-adipocytes and pre-adipocytes differentiation into mature adipocytes. Initially, canonical Wnt-signaling controls the balance of MSC differentiating into myoblasts, osteoblasts or pre-adipocytes [28], and its activation suppresses commitment to the adipocytic lineage, probably suppressing the initiation of adiposity. This is manifested by the fact that genetic deletion of Wnt antagonist Sfrp1 leads to reduction of fat mass concomitant with increased bone mass [29]. On the contrast, our present findings demonstrated that the inflammatory cytokine TNF-α also causally influences adipose dysfunction. Once the adiposity reached the hypertrophic stage after 16-wk HFD feeding, the increased TNF-α activated Wnt/β-catenin signaling and interrupted adipogenesis as indicated by decreased expressions of adipogenic genes, PPARγ and CEBPα, and accompanying with the impaired adipogenesis is excessive adiposity and obesity. Whereas, the genetic ablation of TNF-α mitigate these alterations. These findings indicate that targeting TNF-α and Wnt/β-catenin signaling represents a feasible avenue to attenuate chronic obesity and its related medical complications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare no conflict interest.

Ethics Approval

The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst.

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