Tumor necrosis factor alpha improves glucose homeostasis in diabetic mice independent with tumor necrosis factor receptor 1 and tumor necrosis factor receptor 2

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Abstract. Type 2 diabetes is a serious threat to human health all over the world. It is particularly important to look for the pathogenesis of type 2 diabetes. Researchers have found that obesity was associated with a broad chronic inflammatory response and type 2 diabetes. And tumor necrosis factor alpha (TNF-α) is one of the most important cytokines related with obesity. To explore the functional role of TNF-α in the regulation of glucose homeostasis, TNF-α receptor 1 and TNF-α receptor 2 double knockout (TNFR1/R2 DKO) mouse model were used in our study. After high fat diet (HFD) feeding, we detected that the insulin resistance was dramatically improved and circulated TNF-α was upregulated in TNFR1/R2 DKO mice. Surprisingly, glucose homeostasis was worsened, when we down regulate the levels of plasma TNF-α in TNFR1/R2 DKO mice by administering Adeno associated virus-shRNA-TNF-α (AAV-shTNF-α). Subsequently, in ob/ob mice, we confirmed that the glucose homeostasis could be improved when we up regulate the levels of plasma TNF-α by administering Adeno associated virus-TNF-α (AAV-TNF-α). Our findings suggested that TNFR1 and TNFR2 may not be the only receptors for TNF-α and TNF-α probably plays a positive role in reducing insulin resistance via a TNFRs-independent way in diabetic mice.

Key words: Tumor necrosis factor-α, Diabetes, Insulin resistance, Tumor necrosis factor receptor knockout mice, Obesity

AS TYPE 2 DIABETES has plagued people and caused a large disease burden around the world, there has been an increasing interest on developing treatments for diabetes. Insulin resistance is a well-known and important complication of obesity [1], metabolic syndrome [2, 3] and type 2 diabetes mellitus (T2DM) [2]. Among the various causes of T2DM, inflammation plays a significant role in the development of insulin resistance in obese individuals [4, 5]. Diet-induced obesity (DIO) can induce low-level inflammation and insulin resistance [6, 7]. Among the inflammatory cytokines, TNF-α is one of the key pro-inflammatory cytokines that closely associated with the onset of insulin resistance and T2DM. In the 1990s, the link between TNF-α and insulin resistance in obesity was discovered for the first time in the studies conducted by Hotamisiligil and his colleagues, which found that TNF-α was over-expressed in the adipose tissue of obese animals and humans [1, 8-11]. TNF-α has been implicated in the pathogenesis of insulin resistance and type 2 diabetes because it has been shown to promote several harmful effects in important insulin-responsive tissues, such as skeletal muscle [12] and adipose tissue [11]. At the same time, Researchers found that TNF-α expression was closely related with insulin-stimulated glucose utilization [12-14] and acute TNF-α infusion could inhibit insulin-stimulated glucose utilization [15]. These studies mentioned above showed that TNF-α was found to promote insulin resistance, which is
one of the main causes of diabetes. However, recent studies have shown the controversial results. Namely, that inflammation does not always lead to insulin resistance and anti-inflammatory cannot improve glucose homeostasis. For example, in previous studies, short-term TNF-α inhibition in patients with diabetes or metabolic syndrome did not improve glucose metabolism [16]. In 2014, researchers confirmed that blocking TNF-α signals in fat tissue caused ectopic lipid accumulation which could aggravate diabetic symptoms [17]. In 2015, Zheng Ye’s team reported that depletion of anti-inflammatory Treg cells could increase the insulin sensitivity of adipose tissue [18]. These studies suggested that TNF-α has its positive role in the regulation of glucose metabolism.

TNF signals work in the presence of receptors namely, TNFR1 (p55) and TNFR2 (p75) [19]. TNFR1/R2 knock-out mouse is an irreplaceable model system for exploring the mechanism of blood glucose regulation. Previous studies have found that double knockout of TNFR1 and TNFR2 can improve glucose homeostasis. Studies conducted by Schreyer et al. [20] and Uysal et al. [21, 22] showed that TNFR1/R2 double knockout present alterations in glucose metabolism and insulin sensitivity. In 2006, researchers found that TNFR1/R2 DKO could suppress the development of lipid-induced insulin resistance [23].

Although studies on TNFR1/R2 DKO mice had been performed before, the over-expression of plasma TNF-α in TNFR1/R2 DKO mice was poorly characterized. Our experiments suggested that 1) plasma TNF-α is a key player in glucose homeostasis; 2) TNFR1 and TNFR2 may not be the only receptors of TNF-α, undiscovered receptors for TNF-α may exist.

Materials and Methods

Mice

TNFR1/R2 DKO male mice (p55−/− p75−/−, C57BL/6) were obtained from the Jackson Laboratory (Bar Harbor, ME). Wild-type and ob/ob mice (C57BL/6 background) were purchased from Model Animal Research Center of Nanjing University. To generate the high blood glucose and insulin resistance model, all animals before experiments were fed with a high fat diet (HFD, 60% calories from fat) for 16 weeks, maintained in a 12 h: 12 h light: dark cycle under a controlled temperature between 20 to 25°C, and provided water and food ad libitum provided. This study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Science at Bengbu Medical University. All animal procedures followed the humane treatment guidelines established by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Science at Bengbu Medical University. In this study, all mice were monitored at least twice per day.

RNA preparation and real-time qPCR

For genotyping, 2 mm mouse tail biopsies were collected in a 1.5 mL RNase free microfuge tube. Add 1 mL Trizol reagent used for lysis, put 3 grinding beads, 90 HZ grinding 90 s twice by a grinding miller. Centrifuge for 30 min at 12,000 g at 4°C, transfer the clear supernatant to a new 1.5 mL RNase free tube. Add 0.2 mL of chloroform incubate for 2–3 min, centrifuge for 15 min at 12,000 at 4°C. Transfer the colorless upper aqueous phase to a new tube RNase free tube, add 0.5 mL of isopropanol incubate for 10 min at room temperature. Centrifuge for 10 min at 12,000 g at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. Wash the pellet in 700 uL of 75% ethanol, vortex briefly, then, centrifuge for 5 min at 7,500 g at 4°C. Discard the supernatant, air dry the RNA pellet, dissolved the pellet in 50 uL of RNase free water. Determine the RNA quantity by NanoDrop 2000. cDNA was obtained by total RNA reverse transcription using a cDNA synthesis kit (R223-01, Vazyme, NaiJing), follow the conditions: 25°C 5 min, 42°C 30 min, 85°C 5 min.

Gene expression was analyzed by qPCR machine (LightCycler 480II, Roche) in mix buffer (Q111-02/03, Vazyme). QPCR for cell samples were similar to mouse tails except the grinding step. The levels of mouse mRNA were normalized to 18S as a housekeeping gene. Primers for 18S, P55, P75 and Chop were purchased from Sangon Biotech (Shanghai, China) and were as follows: 18S; Forward: CTGGAAGCCTGGTATGAGGAT Reverse: CAGGGTCAAGAAGTAGGTAGGTGATG; P55; Forward: ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC Reverse: GTC GAT TTC CCA CAA ACA ATG GAG TAG AGC; P75; Forward: ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC Reverse: GTC GAT TTC CCA CAA ACA ATG GAG TAG AGC; P75; Forward: ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC Reverse: GTC GAT TTC CCA CAA ACA ATG GAG TAG AGC; 18S; Forward: CTGGAAGCCTGGTATGAGGAT Reverse: CAGGGTCAAGAAGTAGGTAGGTGATG; P55; Forward: ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC Reverse: GTC GAT TTC CCA CAA ACA ATG GAG TAG AGC; P75; Forward: GAA TAC TAT GAC CAG ACA GCT CAG ATG TGC Reverse: TAT CGC TGG ATG AAG TCG TGT TGG AGA ACG;

Biochemical reagents

D-glucose (158968-100G), insulin (I9278-5ML) were purchased from Sigma. Recombinant mouse TNF-α (CF09) and Adeno-associated virus-shRNA-TNF-α...
Glucose tolerance tests
Mice underwent intraperitoneal glucose tolerance test (GTT) following an overnight fasting (16 h). Before intraperitoneal D-glucose injection (1 g/kg dextrose in 200 μL saline) blood glucose levels were measured from the tail vein blood. Samples were subsequently taken 15, 30, 60, 90, and 120 min after D-glucose administration.

Insulin tolerance tests
Mice underwent insulin tolerance test (ITT) following a 6-h fasting. Before intraperitoneal insulin injection (1.5 U/kg insulin in 200 μL saline), blood glucose levels were measured from the tail vein blood. Samples were subsequently taken 15, 30, 60, 90, and 120 min after insulin injection.

Blood glucose and plasma TNF-α measurements
The blood glucose levels of the mice were measured with a glucose meter (G832631, Roche) after the blood was collected from the tail vein. For ELISA, blood was collected from the tail vein, and plasma was obtained by centrifugation at 2,000 × g, 4°C for 30 min. TNF-α levels in plasma were determined by a mouse TNF-α ELISA kit from R&D systems (MTA00B) according to the manufacturer’s instructions.

AAV injection to mice
AAV introduced to mice through the tail vein injection. The virus was thawed at 25°C before injection, and the desired amount (5*10E9 PFU) of virus was diluted with saline to a final volume of 200 μL per mouse. To easily locate the tail vein, mice were restrained in a mice holder, and their tails were cleaned with a cotton ball soaked in 75% alcohol. The appropriate virus was injected into the tail vein, and to prevent the back flow of virus solution, mild pressure was applied at the injection site immediately after injection until bleeding ceased.

Statistical analysis
Results are presented as the mean ± SEM. Statistical significance was calculated by Student’s t-test, one-way ANOVA or two-way ANOVA. When the ANOVA methods indicated a significant difference among the groups, the significant difference among two groups were compared using a stricter criterion for statistical significance according to the Bonferroni correction; significance was accepted at *p < 0.05.

Results

TNFR1 and TNFR2 double knockout prevent HFD-induced insulin resistance
To study the relationship between inflammation and insulin resistance, a commercial and widely used TNFR1/R2 DKO mice line (p55−/− p75−/−) was adopted in this study. To investigate the deletion efficiency of TNFR1/R2 DKO, we carried out QPCR experiments for genotyping (Fig. 1A, B). After a period of high fat diet feeding, no significant differences in fasted and fed blood glucose levels were observed between TNFR1/R2 DKO and WT mice (Fig. 1C, D). Food intake and body weight also have no significant differences between the two groups (Fig. 1E, F). Previous studies found that glucose metabolism was improved in the DKO mouse model [20-22]. To confirm these findings, we conducted a GTT experiment. As shown in Fig. 1G, there was an increase in the glucose tolerance of DKO mice compared with that in WT HFD mice. Fig. 1H showed the area under the curve (AUC) for GTT. Consistent with this observation, ITT results also revealed an increased insulin sensitivity in DKO mice (Fig. 1I). Fig. 1J shows the AUC for ITT.

The levels of plasma TNF-α were upregulated in TNFR1/R2 DKO mice
To find the reasons of the improvements in glucose metabolism, we measured the plasma TNF-α levels in TNFR1/R2 DKO mice. We detected that the levels of plasma TNF-α in TNFR1/R2 DKO mice exhibited compensatory upregulation compared with WT mice (Fig. 2), suggested that the up-expressed TNF-α in plasma probably relate to the improvement of glucose homeostasis in TNFR1/R2 mice.

TNF-α plays a key role in maintaining glucose homeostasis in TNFR1/R2 DKO mice
Consistent with previous studies, we found that the depletion of TNFR1/R2 in diet induced obese mice could against the onset of glucose metabolism disorders, concurrently, we detected that the expression of plasma TNF-α was upregulated in these DKO mice as a compensation for the loss of TNFR1/R2. These findings evoked
Fig. 1  A. relative mRNA expression of P55 (TNFR1); B. relative mRNA expression of P75 (TNFR2); C. Fasted blood glucose (mmol/L) were measured in WT mice and TNFR1/R2 mice after 16 weeks HFD feeding; D. Fed blood glucose (mmol/L) were measured in WT mice and TNFR1/R2 mice after 16 weeks HFD-feeding; E. Food intake (g/mouse) per day of WT and TNFR1/R2 DKO mice in 2–16 week of HFD-feeding; F. Body weight (g/mouse) of WT and DKO mice; G. GTT were performed in WT mice and TNFR1/R2 mice after 16 weeks HFD-feeding; H. Area under curve (mmol/L*h) for GTT; I. ITT results for WT and TNFR1/R2 mice; J. Area under curve (mmol/L*h) for ITT.
the question of whether high levels of plasma TNF-α play an important role in maintaining glucose metabolism. To study this question, we performed a plasma TNF-α neutralization experiment in DKO mice. We down-regulated the levels of plasma TNF-α in DKO mice by AAV-shTNF-α injection. The control group were injected with AAV-shLacZ. After AAV injection 15 days, the levels of plasma TNF-α in DKO mice was down regulated compare with the control group (Fig. 3A). Food intake, body weight, fasted blood glucose and fed blood glucose were measured separately, no difference between two groups have been found (Fig. 3B–E). However, GTT result in AA V-treated group deteriorated compared with the untreated group (Fig. 3F), AUC for GTT was in Fig. 3G. Consistently, the ITT data after injection increased either (Fig. 3H); AUC for ITT was in Fig. 3I.

AAV-TNF-α injection improved glucose homeostasis in ob/ob mice

To confirm the beneficial role of plasma TNF-α in blood glucose metabolism, 18 weeks old ob/ob mice were injected with AAV-TNF-α to overexpress plasma TNF-α; the control group were injected with AAV-GFP. After AAV injections 10 days, food intake and body weight were have no difference between two groups (Fig. 4A, B). Fasted and fed blood glucose were no difference either (Fig. 4C, D). However, the levels of plasma TNF-α in AAV-TNF-α injected mice were upregulated compared with AAV-GFP injected mice (Fig. 4E). The expression of TNFR1 and TNFR2 in ob/ob mice before and after AAV injection have been no significant differences (Supplementary Fig. 1A–D). Subsequently, the GTT experiment revealed a significant improvement in the AAV-TNF-α injected group (Fig. 4F), AUC for GTT was in Fig. 4G. ITT results were consistent with GTT results (Fig. 4H, I).

Discussion

Type 2 diabetes is considered as an inflammatory disease [24]. Evidence from previous studies found that obesity-associated insulin resistance was related with increased concentrations of inflammatory cytokines in the circulation of both obese rodent models and humans. TNF-α stands out and has been considered as the pathogenesis of insulin resistance [12, 14]. However, there are several clinical studies in which patients have received an anti-TNF-α therapy to treat insulin resistance [25-27], and the results obtained during these trials seem to be controversial. These suggested that the relationship between inflammation and insulin resistance is much more complicated than previously thought, and several discoveries confirmed that pro-inflammation factors probably have a positive role in the regulation of blood glucose [16-18].

Our study found that depleting TNFR1/R2 improved the regulation of glucose homeostasis, which was consistent with the achievements of other researchers [9, 23]. What’s more, we detected that the levels of plasma TNF-α in TNFR1/R2 DKO mice exhibited compensatory upregulation. In the following experiments, we decreased plasma TNF-α levels in TNFR1/R2 DKO mice via AAV-shTNF-α injection and found that the improvement of blood glucose homeostasis was blunted. These GTT and ITT phenomena suggested that the improvements in blood glucose homeostasis observed after TNFR1/R2 knockout was not due to the inhibition of TNF-α/TNFRs signal transduction. After AAV injection, the plasma TNF-α were down-regulated, this may decrease the binding between TNF-α and all its receptors, including TNFRs or other uncovered receptors. Moreover, AAV-shTNF-α treatment diminishes the improvement in glucose homeostasis caused by the depletion of the TNFRs. Thus, it can be inferred that, in addition to TNFR1 and TNFR2, other receptors probably be involved and that their interaction with TNF-α plays a role in improving glucose homeostasis.

We also found that the regulation of glucose homeostasis was improved by overexpressing plasma TNF-α in the ob/ob mice by AAV-TNF-α injection. Thus, it can be deduced that the pro-inflammatory TNF-α plays a positive role in the process of blood glucose regulation. TNF-α/TNFRs signaling pathway may not be the only TNF-α
Fig. 3  A. Blood TNF-α levels (pg/mL) in AAV-shLacZ or AAV-shTNF-α injected DKO mice; B. Food intake (g/mouse) per day of DKO mice after AAV-shLacZ or AAV-shTNF-α injection (5*10^9 PFU); C. Body weight of DKO mice after AAV-shLacZ or AAV-shTNF-α injection (5*10^9 PFU); D. Fasted blood glucose of DKO mice after AAV-shLacZ or AAV-shTNF-α injection (5*10^9 PFU); E. Fed blood glucose of DKO mice after AAV-shLacZ or AAV-shTNF-α injection (5*10^9 PFU); F. GTT results for DKO mice after AAV-shLacZ or AAV-shTNF-α injection 18 days (5*10^9 PFU); G. Area under curve (mmol/L*h) for GTT; H. ITT results for DKO mice after AAV-shLacZ or AAV-shTNF-α injection 20 days (5*10^9 PFU); I. Area under curve (mmol/L*h) for ITT.
Fig. 4  A. Food intake (g/mouse) per day of ob/ob mice after AAV-GFP or AAV-TNF-α injection (5*10E9 PFU); B. Body weight of ob/ob mice after AAV-GFP or AAV-TNF-α injection (5*10E9 PFU); C. Fasted blood glucose of ob/ob mice after AAV-GFP or AAV-TNF-α injection (5*10E9 PFU); D. Fed blood glucose of ob/ob mice after AAV-GFP or AAV-TNF-α injection (5*10E9 PFU); E. Blood TNF-α levels (pg/mL) in ob/ob mice after AAV-GFP or AAV-TNF-α injection (5*10E9 PFU); F. GTT results for ob/ob mice after AAV-GFP or AAV-TNF-α injection 13 days (5*10E9 PFU); G. Area under curve (mmol/L*h) for GTT; H. ITT results for ob/ob mice after AAV-GFP or AAV-TNF-α injection 15 days (5*10E9 PFU); I. Area under curve (mmol/L*h) for ITT.
signaling pathway, different receptors probably play a role in blood glucose regulation in individuals with insulin resistance. Although the level of plasma TNF-α in ob/ob mice were higher than wild-type mice [28], ob/ob mice were still in the state of insulin resistance. The reason might be that there was "cytokines resistance" in diabetic mice and the "cytokines resistance" lead to TNF-α benefit function blocked in ob/ob mice. When we over regulated the plasma TNF-α to a higher level and compensated the "cytokines resistance" phenomenon, the insulin resistance was improved.

Consider the findings of our study, our traditional views about TNF-α should be updated. Indeed, the process of the inflammatory effects of TNF-α on blood glucose is complex. Except for the negative effects, TNF-α may be beneficial to blood glucose metabolism. Maybe we can conclude that TNF-α probably plays a dual role in glucose metabolism. And in addition to TNF-α receptor 1 and receptor 2, there may be other TNF-α receptors participating in the process of glucose metabolism. Since the presence of novel TNF-α receptors cannot be conclusively determined based on our currently available evidences, further studies are needed.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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