RESEARCH

TNF signaling impacts glucagon-like peptide-1 expression and secretion

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Abstract

Numerous studies have implicated tumor necrosis factor α (TNFα) in the pathogenesis of type 2 diabetes. However, the role of its primary receptor, TNF receptor 1 (TNFR1), in homeostatic regulation of glucose metabolism is still controversial. In addition to TNFα, lymphotoxin α (LTα) binds to and activates TNFR1. Thus, TNFα and LTα together are known as TNF. To delineate the role of TNF signaling in glucose homeostasis, the present study ascertained how TNF signaling deficiency affects major regulatory components of glucose homeostasis. To this end, normal diet-fed male TNFR1-deficient mice (TNFR1−/−), TNFα/LTα/LTβ triple-deficient mice (TNF/LT∆3) and their littermate controls were subjected to intraperitoneal glucose tolerance test, insulin tolerance test and oral glucose tolerance test. The present results showed that TNFR1−/− and TNF/LT∆3 mice vs their controls had comparable body weight, tolerance to intraperitoneal glucose and sensitivity to insulin. However, their tolerance to oral glucose was significantly increased. Additionally, glucose-induced insulin secretion assessments revealed that TNFR1 or TNF/LT deficiency significantly increased oral but not intraperitoneal glucose-induced insulin secretion. Consistently, qPCR and immunohistochemistry analyses showed that TNFR1−/− and TNF/LT∆3 mice vs their controls had significantly increased ileal expression of glucagon-like peptide-1 (GLP-1), one of the primary incretins. Their oral glucose-induced secretion of GLP-1 was also significantly increased. These data collectively suggest that physiological TNF signaling regulates glucose metabolism primarily through effects on GLP-1 expression and secretion and subsequently insulin secretion.

Introduction

With a focused effort of more than two decades, it has been well established that inflammation plays a crucial role in the pathogenesis of type 2 diabetes mellitus (Hotamisligil 2017). Tumor necrosis factor-alpha (TNFα) is one of the prototype pro-inflammatory cytokine and an essential component of various inflammatory responses (Vinay & Kwon 2012). Indeed, the amelioration of over-nutrition-induced insulin resistance and glucose
intolerance by treatment with anti-TNFα is one piece of the earliest evidence that inflammation plays a role in the pathogenesis of type 2 diabetes mellitus (Hotamisligil et al. 1993). Since then, numerous studies have demonstrated that TNFα causes insulin resistance and consequently impairs glucose tolerance, which is widely believed to be dependent on TNFα-induced serine phosphorylation of insulin receptor substrates (IRSs) (Hotamisligil et al. 1993). In addition, the increased tolerance to glucose in TNFα−/− mice has been shown to coincide with decrease in body weight and adiposity (Ventre et al. 1997). As obesity may cause various abnormalities in homeostatic regulation of glucose metabolism, TNFα is believed to influence glucose homeostasis through an obesity-dependent manner too. Furthermore, several studies have shown that TNFα reduces insulin secretion by cultured pancreatic β cell lines such as INS-1 (Zhang & Kim 1995) and HIT-T15 (Tsiotra et al. 2001), suggesting that in addition to insulin resistance, it may also impact glucose metabolism through effects on insulin secretion.

Glucagon-like peptide-1 (GLP-1) is one of the primary incretins that is primarily produced by intestinal enteroendocrine L-cells (Drucker 2018). It may enhance insulin secretion in a glucose-dependent manner and subsequently lower blood glucose level (Drucker 2018). More recently, TNFα has been shown to reduce GLP-1 expression and secretion in cultured NCI-H716 cells (Gagnon et al. 2015). Furthermore, treatment with etanercept, an anti-TNF biologic, increases GLP-1 secretion by cultured adult mouse intestinal cells isolated from high-fat diet-fed mice (Gagnon et al. 2015). These data collectively suggest that TNFα may impact insulin secretion and glucose homeostasis through a GLP-1-dependent mechanism.

By far, two receptors of TNFα, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), have been identified. Of them, TNFR1 is essential for the most biological activities of TNFα (Al-Lamki & Mayadas 2015, Probert 2015). In contrast to the relatively consistent role of TNFα in body weight and glucose homeostasis, the role of TNFR1 in body weight and glucose homeostasis is largely controversial. Like TNFα−/− mice, TNFR1−/− mice were ever shown to have decreased body weight (Martins et al. 2017). However, they had paradoxically increased adiposity and normal glucose tolerance (Martins et al. 2017). Furthermore, there was another report showing that TNFR1 deficiency impacted neither mouse body weight nor glucose homeostasis (Toda et al. 2010). Given that anti-TNF biologics are one group of the most frequently prescribed drugs for patients of various inflammatory diseases (Antonopoulos et al. 2017); further studies are needed to delineate the role of TNFR1 in body weight and glucose homeostasis in a non-diabetic setting.

In addition to TNFα, the TNFR1 is bound and activated by lymphotoxin α (LTα), one of the close homologs of TNFα. Analyses on TNFα- and/or LTα-deficient mice have demonstrated that each of them has unique and shared functions (Gubernatorova & Tumanov 2016). Therefore, TNFα and LTα together are frequently referred to as TNF. We recently showed that TNFα/LTα/LTβ triple deficiency (TNF/LTα) did not significantly impact body weight or glucose tolerance (Hu et al. 2017). This is in direct contrast to the above-mentioned phenotype of TNFα−/− mice, suggesting a functional difference between TNFα and TNFα, thus warranting further investigation on the role of TNF signaling in glucose metabolism. In the present study, we therefore examined the effect of TNFR1 and TNF/LT deficiency on glucose homeostasis and its regulatory components, with a focus on the GLP-1 system. Our results showed that physiological TNF signaling influences glucose homeostasis primarily by effecting gut expression and secretion of GLP-1.

Materials and methods

Animals

University of Maryland, Baltimore (UMB) is an AAALAC accredited institution. All procedures of this study were approved by the Institutional Animal Care and Use Committee at UMB, and all the animals were treated humanely and with regard for alleviation of suffering. Male TNFR1−/− (Stock#: 002818) and TNF/LTα−/− (Stock#: 005108) mice were obtained from the Jackson Laboratory. They were crossed with female C57Bl/6J mice to generate TNFR1−/− and TNF/LTα−/− mice respectively. The TNFα−/− and its littermate TNFα−/− control (WT) used in the present study were generated through inbreeding of TNFα−/−, and the TNF/LTα and its littermate TNF/LTα−/− control (WT) were generated through inbreeding of TNF/LTα−/−.

Intraperitoneal glucose tolerance test

Before testing, mice were fasted for 16 h. On the day of experiments, after determination of basal blood glucose level using an automatic glucometer (Glucotrend 2, Roche Diagnostics), mice were intraperitoneally injected with glucose (2 g/kg body weight). Blood glucose levels at 15, 30, 60 and 120 min after injection were then measured using the automatic glucometer.
Oral glucose tolerance test

Before testing, mice were fasted for 16 h. On the day of experiment, following determination of basal blood glucose level using an automatic glucometer (Glucotrend 2, Roche Diagnostics), mice were orally gavaged with glucose (2g/kg body weight). Blood glucose levels at 15, 30, 60 and 120 min after injection were then measured as described above.

Insulin tolerance test

Before testing, mice were fasted for 4h. After determination of basal blood glucose level using an automatic glucometer (Glucotrend 2, Roche Diagnostics), mice were intraperitoneally injected with insulin (0.5U/kg body weight). Blood glucose levels at 15, 30, 60 and 120 min after injection were then measured as described earlier.

Determination of blood insulin, GLP-1 and GIP levels

When performing intraperitoneal glucose tolerance test (IPGTT) and oral glucose tolerance test (OGTT), sera were harvested at 0, 15 and 30 min after intraperitoneal injection or oral gavage of glucose, and insulin levels in these sera were determined using Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Elk Grove Village, IL, USA) per manufacturer’s instructions. To assess glucose-induced secretion of GLP-1 and GIP, TNFR1−/−, TNF/¡/−¡ and their littermate controls were fasted for 16 h, followed by oral gavage of glucose (2 g/kg body weight). Mice were then killed for harvesting plasma at 0, 15 and 30 min after oral gavage of glucose. Plasma were obtained in the presence of EDTA (10% of blood), DPP4 inhibitor (10 µL/mL blood, Millipore) and protease and phosphatase inhibitor mini table (0.00987 g/mouse, Thermo Fisher). GLP-1 (High Sensitivity GLP-1 Active Chemiluminescent ELISA Kit, EMD Millipore Corporation) and GIP (RAT/MOUSE GIP (TOTAL) ELISA KIT, EMD Millipore Corporation) levels in these plasmas were determined per manufacturer’s instructions.

Real-time RT-PCR

Duodenal and ileal villi were obtained from the proximal duodenum and ileum by scraping with mild pressure from the short edge of a glass slide as previously described (Jang et al. 2007). The isolated tissues were washed three times with ice-cold PBS, and then aliquoted and stored at −80°C. Total RNA was extracted from these purified villi using the TRIzol reagent (Invitrogen). The quality of RNA was assessed by determination of the ratio of absorbance at 260 nm to absorbance at 280 nm by a NanoDrop. Two micrograms of total DNase-treated RNA were reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystem) per manufacture’s instruction. Real-time PCR was performed using LightCycler 480 SYBR Green I Master in the LightCycler (Roche). Reactions were performed in a total volume of 10 µL containing 1 µL cDNA, 0.2 µM of each primer and 5 µL of the SYBR Green reaction mix. The amplification protocol was as follows: 95°C/5 min (95°C/10 s, 60°C/20 s and 72°C/30 s) × 45. Following amplification, a dissociation curve analysis was performed to insure purity of PCR product. The specific sense and antisense primers are as follows: GIP: 5′-TGA GTT CCG ATC CCA TGC TAA-3′ and 5′-CCA GTC CCA GTA TTC TG-3′; GLP-1: 5′-TTA CTT TGT GGC TGG ATT GCT T-3′ and 5′-AGT GGC GTT GTG CTT CAT TCA-3′ and GAPDH: 5′-TGA ACG GGA AGC TCA CTG G-3′ and 5′-TCC ACC ACC CTG TTG CTG G-3′.

Immunohistochemistry

Formalin-fixed ileum were embedded in paraffin. Fifteen slides of 5 µm tissue sections (3 sections/slide) per animal were obtained, and one of every three slides were used for immunohistochemistry. Following heat-induced antigen retrieval using antigen retrieval solution (Dako), tissue sections were stained with anti-GLP-1 (Abcam). Briefly, tissue sections were incubated with anti-GLP-1 overnight at 4°C followed by washing, incubation with HRP-conjugated secondary antibody for 1 h at room temperature, visualization using DAB chromogen solution and counterstaining. Staining without any primary antibody was used as negative controls.

Statistics

All data are expressed as mean±s.e.m. unless noted otherwise. Statistical tests were performed using two-way analysis of variance (ANOVA) or unpaired student t test using GraphPad Prism (version 5; GraphPad Software). The significance level was set at P<0.05.

Results

TNF signaling deficiency increases mouse tolerance to oral but not intraperitoneal glucose

TNFα deficiency has been shown to decrease mouse body weight and adiposity (Ventre et al. 1997), suggesting TNF signaling may impact homeostatic regulation of glucose
metabolism through an obesity-dependent manner. However, we recently demonstrated that normal diet-fed 7-month-old TNF/LT\textsuperscript{−/−} and control mice did not have any significant difference in the weights of the body and any examined adipose tissues (Hu et al. 2017). To further document whether TNF signaling impacts glucose homeostasis through the obesity-dependent mechanism, we compared the weights of body and adipose tissues between normal diet-fed TNFR1\textsuperscript{−/−} and littermate control mice. Table 1 shows that TNFR1 deficiency altered neither the body weight nor the weights of subcutaneous, epididymal and perirenal adipose tissues at the age of 25 weeks. As such, in the present study, normal diet-fed TNFR1\textsuperscript{−/−}, TNF/LT\textsuperscript{−/−} and their littermate control mice under the age of 25 weeks were used to further investigate whether TNF signaling impacts glucose homeostasis through an obesity-independent manner.

To delineate the role of TNF signaling in glucose homeostasis, we first performed IPGTT on TNFR1\textsuperscript{−/−} and littermate controls. Figure 1A reveals that TNFR1 deficiency did not significantly influence the metabolism of glucose administered through intraperitoneal injection. We neither observed any significant effect of TNFR1 deficiency on mouse sensitivity to exogenous insulin (Fig. 1B). Unexpectedly, TNFR1\textsuperscript{−/−} mice vs littermate controls had significantly increased tolerance to glucose administered through oral gavage (Fig. 1C). Consistently, TNF/LT\textsuperscript{−/−} mice and littermate controls had comparable responses to intraperitoneally injected glucose and insulin (Fig. 1D and E), whereas TNF/LT\textsuperscript{−/−} mice vs littermate controls had significantly increased tolerance to orally gavaged glucose (Fig. 1F). These data collectively suggest that physiological TNF signaling may impact homeostatic regulation of glucose metabolism through a gut-dependent mechanism.

**TNF signaling deficiency influences oral but not intraperitoneal glucose-induced insulin secretion**

The metabolism of glucose is determined by both circulating insulin level and sensitivity to insulin. Since we observed that neither TNFR1 nor TNF/LT deficiency significantly altered sensitivity to insulin (Fig. 1B and E), we next assessed their effects on glucose-induced insulin secretion. Consistent with the above blood glucose assessments, TNFR1 or TNF/LT deficiency did not alter intraperitoneal glucose-induced insulin secretion (Fig. 2A and C), whereas significantly increased oral glucose-induced insulin secretion (Fig. 2B and D), strongly supporting that physiological TNF signaling regulates insulin secretion and thus glucose homeostasis through a gut-dependent manner.

**Table 1** The weight of body and adipose tissues of male 25-week-old TNFR1\textsuperscript{−/−} and littermate control (WT) mice.

<table>
<thead>
<tr>
<th></th>
<th>Body</th>
<th>Subcutaneous</th>
<th>Epididymal</th>
<th>Perirenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (g, n=8)</td>
<td>26.9±2.3</td>
<td>0.2±0.03</td>
<td>0.47±0.09</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td>TNFR1\textsuperscript{−/−} (g, n=7)</td>
<td>27.1±2</td>
<td>0.18±0.02</td>
<td>0.45±0.1</td>
<td>0.17±0.06</td>
</tr>
<tr>
<td>P value (t test)</td>
<td>0.87</td>
<td>0.39</td>
<td>0.92</td>
<td>0.48</td>
</tr>
</tbody>
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**Figure 1**

TNF signaling deficiency increases mouse tolerance to orally gavaged but not intraperitoneally injected glucose. (A, B and C) TNFR1\textsuperscript{−/−} and littermate controls (10–16 weeks old, n=6/group) were subjected to IPGTT (A), ITT (B) and OGTT (C). The area under curve (AUC) of OGTT was presented in the inset. (D, E and F) TNF/LT\textsuperscript{−/−} and littermate controls (10–18 weeks old, n=6/group) were subjected to IPGTT (D), ITT (E) and OGTT (F). The AUC of OGTT was presented in the inset. *P<0.05 vs litter controls (WT), two-way ANOVA or student’s t test (AUC).
TNF signaling deficiency increases GLP-1 expression in intestinal L-cells

GLP-1 and GIP are two primary incretins produced by the gut in response to glucose ingestion, and TNF signaling has been shown to impact GLP-1 expression in cultured intestinal enteroendocrine L-cells (Gagnon et al. 2015). We therefore assessed GIP and GLP-1 mRNA expression levels in the duodenal and ileal villi of TNFR1−/−, TNF/LT−/−, and their littermate control mice. Consistent with previous reports (Svendsen & Holst 2016), GIP mRNA appeared to be expressed relatively higher in the duodenal villi, whereas GLP-1 mRNA appeared to be expressed higher in the ileal villi (Fig. 3A vs B and C vs D). TNFR1 (Fig. 3A and B) or TNF/LT (Fig. 3C and D) deficiency did not alter GIP mRNA expression in the duodenal and ileal villi. In contrast, while TNFR1 or TNF/LT deficiency did not change GLP-1 mRNA expression in the duodenal villi, they significantly increased GLP-1 mRNA expression in the ileal villi. To further document the effect of physiological TNF signaling on ileal GLP-1 expression, ileal GLP-1 protein was visualized through immunohistochemistry. Fig. 4B and E show that TNFR1 and TNF/LT deficiency did not significantly change the number of GLP-1+ cells in the ileum, but significantly increased GLP-1 protein expression in the ileum (Fig. 4C and F).

TNF signaling deficiency increases oral glucose-induced secretion of GLP-1 but not GIP

Given the increased intestinal expression of GLP-1 but not GIP in TNFR1−/− and TNF/LT−/− mice, we next examined whether TNF signaling deficiency influences GLP-1 secretion in response to oral gavage of glucose. Fig. 5A and C reveals that TNFR1 or TNF/LT deficiency was sufficient to significantly increase oral glucose-induced GLP-1 secretion. In contrast, neither TNFR1 nor TNF/LT deficiency significantly altered the secretion of GIP in response to oral gavage of glucose (Fig. 5B and D).

Discussion

Since TNFα neutralization was shown to ameliorate obesity-linked insulin resistance more than two decades ago (Hotamisligil et al. 1993), numerous studies have demonstrated the specialized role of TNF signaling in the...
pathogenesis of insulin resistance and thus type 2 diabetes (Liu et al. 2016). In contrast, its role in the physiological regulation of glucose homeostasis remains controversial. For instance, while normal diet-fed TNFα−/− mice vs WT controls were first shown to have decreased fasting glucose and insulin levels (Ventre et al. 1997), indicative of improvement in insulin sensitivity and glucose tolerance, one most recent report showed that they had normal insulin sensitivity and glucose tolerance (Li et al. 2016). In the present study, to ascertain the physiological role of TNF signaling in homeostatic regulation of glucose metabolism, we investigated the effect of TNFR1 or TNF/LT deficiency on the regulatory components of glucose homeostasis. The major findings include that (1) TNFR1 or TNF/LT deficiency did not significantly alter mouse body weight and adiposity in early adulthood; (2) TNFR1 or TNF/LT deficiency influences mouse tolerance to oral but not intraperitoneal glucose; (3) TNFR1 or TNF/LT deficiency did not influence mouse sensitivity to exogenous insulin and intraperitoneal glucose-induced insulin secretion, but increased oral glucose-induced insulin secretion; (4) TNFR1 or TNF/LT deficiency increased intestinal expression and oral glucose-induced secretion of GLP-1 but not GIP.
GLP-1 is one of the two primary incretins (GIP is the other primary incretin) secreted by the intestine on ingestion of glucose or nutrients to stimulate insulin secretion from pancreatic β cells (Seino et al. 2010). The success of GLP-1 receptor agonists and DPP4 inhibitors in treatment of type 2 diabetes has fully manifested its crucial role in homeostatic regulation of glucose metabolism (Taylor 2018). As such, the most important finding in the present study is that physiological TNF signaling regulates gut GLP-1 expression and secretion. This is consistent with one recent study showing that TNFα treatment decreased GLP-1 expression and secretion in cultured L-cells (Gagnon et al. 2015). Collectively, these data furthermore suggest that TNF signaling may directly act on GLP-1 producing cells. However, further studies, particularly those using conditional TNF signaling deficient mice, are required to rule out the possibility that TNF signaling indirectly regulates GLP-1 expression. Notably, this recent study also showed that treatment with etanercept in high-fat diet-fed mice increased GLP-1 secretion by their intestinal cells that were isolated and cultured, suggesting that the downregulation of GLP-1 expression and secretion by TNF signaling may play a pathophysiological role. However, given that the impairment of incretin response in type 2 diabetes is believed to be attributable primarily to defect in GIP but not GLP-1 system (Nauck & Meier 2016), caution should be taken when extrapolating the physiological function of TNF signaling to the pathogenesis of type 2 diabetes. Further studies are definitely required to verify this potential pathophysiological role of TNF signaling.

It is noteworthy that the present data also strongly suggest that in contrast to its multiple pathophysiological actions on glucose homeostasis (Liu et al. 2016), the physiological action of TNF signaling on homeostatic regulation of glucose metabolism is almost completely dependent on effecting the gut production of GLP-1. This is clearly evidenced by that in normal diet-fed mice, TNFR1 or TNF/LT deficiency does not impact insulin sensitivity and tolerance to glucose administered through intraperitoneal injection (Fig. 1). These results are consistent with one previous study showing that TNFR1 deficiency does not affect insulin sensitivity in normal diet-fed mice (Toda et al. 2010). Interestingly, this specialized role of GLP-1 in homeostatic regulation of glucose metabolism by physiological TNF signaling is reminiscent of its emerging role in homeostatic regulation of glucose metabolism by the gut microbiota (Everard & Cani 2014, Zhao et al. 2018). Given the well-established role of TNF signaling in inflammation (Vinay & Kwon 2012) and the latter in the regulation of glucose homeostasis by the gut microbiome (Rajani & Jia 2018), further studies are warranted to examine whether this downregulation of GLP-1 expression and secretion by TNF signaling plays a role in the regulation of glucose metabolism by the gut microbiota.

Obesity is one of the well-established risk factors for insulin resistance and type 2 diabetes. TNFα deficiency has been shown to decrease fasting glucose and insulin levels and meanwhile reduce body weight and adiposity in both obese and non-obese mice (Ventre et al. 1997, Li et al. 2016), suggesting that TNF signaling may impact glucose homeostasis through an obesity-dependent mechanism. However, anti-TNF therapy has been shown to improve glucose intolerance but result in a weight-gain in human patients (Peluso & Palmery 2016), casting doubt on this specialized role of obesity in glucose homeostatic regulation by TNF signaling. In the present study, we found that deficiency of either TNFR1 or TNF/LT did not significantly alter the body weight and adiposity of normal diet-fed mice. This is consistent with one previous study showing that TNFR1 deficiency does not impact mouse body weight (Toda et al. 2010). Notably, there is also another study showing that TNFR1 deficiency decreased mouse body weight, but paradoxically increased adiposity (Martins et al. 2017). As such, although further studies are still needed to address the inconsistency in the body weight and adiposity effects of TNF signaling deficiency, all these studies have supported that the obesity-dependent mechanism may be trivial for the physiological role of TNF signaling in homeostatic regulation of glucose metabolism.

Although the present study provides compelling evidence that TNF signaling physiologically regulates GLP-1 expression and secretion and thus impacts glucose homeostasis, there are several significant limitations that need to be addressed by further studies. First, we did not determine whether GLP-1 blocking or deficiency abolishes the glucose metabolic effect of physiological TNF signaling. Secondly, we did not investigate whether this downregulation of GLP-1 by TNF signaling plays a role in various pathophysiologyes, particularly obesity and type 2 diabetes. This will require exploiting both obesity or type 2 diabetes animal models and various genetically modified mice. Thirdly, both TNFR1 and TNF/LT deficiency reflects TNF signaling as a whole. The respective roles of TNFα and LTα have to be determined. This will require investigation on mice deficient in TNFα or LTα only. However, the demonstration that one gene’s genetic modification impacts the other gene’s expression due to their proximity on the DNA (Kuprash et al. 2005)
suggests that the use of TNFα−/− and LTα−/− mouse models may not be enough. Fourthly, although the normal body weights of TNFRI−/− and TNF/LT-deficient mice strongly suggest that TNF signaling deficiency may not impact glucose absorption, further studies are needed to confirm this.

**Conclusion**

The present study demonstrates that the physiological TNF signaling contributes to homeostatic regulation of glucose metabolism primarily through effects on GLP-1 expression and secretion and subsequently insulin secretion.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**

S C, W W, M C, X Q, L Q and L Z acquired and analyzed the data used in the present study. Q C and Z Y interpreted the present results. S C and Z Y drafted the manuscript. W W, M C and Q C were also major contributors in writing the manuscript. All authors read and approved the final manuscript.

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