Loss of \textit{Tbk1} kinase activity protects mice from diet-induced metabolic dysfunction

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\textbf{ABSTRACT}

Objective: TANK Binding Kinase 1 (TBK1) has been implicated in the regulation of metabolism through studies with the drug amlexanox, an inhibitor of the IκB kinase (IKK)-related kinases. Amlexanox induced weight loss, reduced fatty liver and insulin resistance in high fat diet (HFD) fed mice and has now progressed into clinical testing for the treatment and prevention of obesity and type 2 diabetes. However, since amlexanox is a dual IKKε/TBK1 inhibitor, the specific metabolic contribution of TBK1 is not clear.

Methods: To distinguish metabolic functions unique to TBK1, we examined the metabolic profile of global \textit{Tbk1} mutant mice challenged with an obesogenic diet and investigated potential mechanisms for the improved metabolic phenotype.

Results and conclusion: We report that systemic loss of TBK1 kinase function has an overall protective effect on metabolic readouts in mice on an obesogenic diet, which is mediated by loss of an inhibitory interaction between TBK1 and the insulin receptor.

Keywords TBK1; IKKe; Obesity; Insulin; Insulin resistance; Metabolism

1. INTRODUCTION

The prevalence of obesity has expanded dramatically over the last 30 years worldwide and has contributed to the increasing health burden of associated complications, including insulin resistance, type 2 diabetes, cardiovascular disease, hypertension, liver steatosis and dyslipidemia [1—3]. A primary pathology of obesity and metabolic disorders is the induction of chronic low-grade, unresolved inflammation in organs pertinent to energy homeostasis. This so-called “meta-inflammation” consists of increased levels of proinflammatory cytokines and macrophage infiltration in white adipose tissue (WAT) and is coincident with the manifestation of insulin resistance [4—6]. While the etiology of insulin resistance is complicated and multifaceted, numerous studies have demonstrated that blocking inflammatory mediators through genetic or pharmacological means results in improved insulin and glucose tolerance [7—12].

NF-κB is a major transcriptional driver of inflammation that is activated in response to cytokines and pathogenic stimuli to promote the upregulation of inflammatory and immune regulatory gene expression. Not surprisingly, NF-κB is chronically active in many inflammatory diseases and over the past two decades has gained significant attention in the metabolism field due to its high-level activity in affected tissues [13,14]. Most metabolic studies have centered on canonical NF-κB activators, such as IKKα and IKKβ; however, Chiang and colleagues [14] reported that the noncanonical activators, IKKe and TBK1, are more highly expressed than canonical activators in metabolically affected tissues of obese animals. Subsequent investigations revealed that \textit{Ikbke}\textsuperscript{−/−} mice and mice treated with amlexanox (a dual IKKe/TBK1 small molecule inhibitor) are protected from diet-induced obesity (DIO) and associated metabolic syndrome conditions [14,15]. This protection is thought to result from loss of TBK1 and IKKe metabolic functions in repressing adaptive energy expenditure in a diet-driven inflammatory state. Due to the embryonic lethality of \textit{Tbk1}\textsuperscript{−/−} mice and the nature of amlexanox as a dual IKKe and TBK1 inhibitor, distinguishing metabolic functions unique to TBK1 remains a challenge [16]. As amlexanox enters the clinical space for the treatment of obesity and type 2 diabetes in human trials, it has become increasingly important to understand the implications of systemic TBK1 inhibition during metabolic stress.

We set out to investigate TBK1-dependent contributions to metabolism in a metabolically challenged rodent model. We did this utilizing a \textit{Tbk1} mutant mouse that harbors two copies of a null \textit{Tbk1} allele (\textit{Tbk1}\textsuperscript{fl/fl}) [17]. This particular “null allele” encodes a truncated TBK1 protein that is catalytically inactive and expressed at low levels, thereby allowing analysis of global TBK1 kinase loss in vivo. Recently, Zhao and colleagues [18] reported the metabolic effects of an adipocyte specific \textit{Tbk1}-deficient animal model. Here, we focus on the analysis of a global loss-of-function mutant, which provides an opportunity to evaluate the function of TBK1 in a wider context than just the adipocyte.

We report that \textit{Tbk1}\textsuperscript{fl/fl} mice are resistant to high fat diet-induced weight gain and pancreatic islet hyperplasia and hypertrophy. Additionally, we found that TBK1 inhibits insulin receptor signaling in the HFD setting, a function that is absent in \textit{Tbk1}\textsuperscript{−/−} animals. As a result,
HFD-fed Tbk1−/−/D mice are more responsive to insulin compared to Tbk1+/+ mice, as seen by their more efficient glucose absorption, greater AKT activity and higher levels of insulin receptor substrate (IRS) protein. Accordingly, Tbk1−/+-/D mice are more active and have greater energy expenditure relative to Tbk1+/+ mice, accounting for their leaner body composition. Consistent with an improved metabolic phenotype, Tbk1−/+-/D mice also display reduced inflammation in liver and WAT compared to Tbk1+/+ mice post HFD. Taken together, our results describe a healthier phenotype with global loss of TBK1 kinase function under metabolic challenge and highlight the suitability of TBK1 as a therapeutic target in the prevention and treatment of type 2 diabetes and obesity.

2. MATERIALS AND METHODS

2.1. Animals

Tbk1−/+-/D mice were generated in a pure 129S5 strain as previously described [17] and were generously provided by Pfizer (Cambridge, MA). Tbk1−/+-/D mice, Tbk1−/+ mice and C57BL/6 mice were bred and maintained in a pathogen-free barrier facility with access to food and water ad libitum. All protocols for mouse use and euthanasia were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center (Dallas, TX).

2.2. Animal studies

All experiments were conducted using littermate-controlled male mice. HFD experiments were initiated at 4 weeks of age. Mice were fed a normal chow diet (16% protein diet, irradiated) (Teklad Global Diets;Envigo, East Millstone, NJ), or a 60% HFD (D12492; Research Diets, Inc., New Brunswick, NJ). Tissue and blood were collected in the fasted state. Before the metabolic cage studies, the mice were housed individually in metabolic chambers for 1 week for acclimation. Metabolic measurements were obtained continuously using TSE metabolic chambers (TSE Labmaster System; TSE Systems, Bad Homburg, Germany) in an open-circuit indirect calorimetry system where the mice had access to water and either ND or 60% HFD ad libitum. The fat mass and the bone-free lean body composition were measured in nonanesthetized mice using an Echo 3-in-1 nuclear magnetic resonance (MRI) mini Spec instrument (Bruker, Rheinstetten, Germany). For oral glucose tolerance tests (OGTTs), mice were fasted for 4 h prior to administration of glucose (2 g/kg body weight by oral gavage). Mice did not have access to food throughout the experiment. Blood from the tail was measured for glucose content using Contour glucometer strips. For insulin signaling assays, mice were fasted overnight for insulin injection the next day by i.p. (1 U/kg body weight, insulin). At 15 min post insulin injection, mice were sacrificed and tissues (liver, subcutaneous WAT, skeletal muscle) were harvested and immediately snap-frozen in liquid nitrogen.

2.3. RNA isolation and quantitative RT-PCR

Tissues were excised from mice and snap-frozen with liquid nitrogen. Total RNA was isolated after tissue homogenization in TRIzol (Thermo Fisher, Waltham, MA) and RNA extraction was performed using an RNeasy RNA extraction kit (Qiagen, Germantown, MD). RNA was quantified using a NanoDrop instrument (Thermo Fisher). Complementary DNA was prepared by reverse transcribing 1 μg of RNA with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Supplementary Table 1 lists the primer sets used for quantitative RT-PCR. qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad), with iQ SYBR Green Supermix (Bio-Rad). Results were measured using the comparative threshold cycle (Ct) method, with β-actin used for normalization. Fold changes and statistical significance were calculated from three independent replicates.

2.4. Immunoblotting

Tissues were lysed in ice-cold RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS).
containing cocktails of protease (Thermo Fisher Scientific) and phosphatase inhibitors (Sigma—Aldrich, St. Louis, MO) and centrifuged for 20 min at 13,000 \( \times \) g at 4 °C. Total protein concentration was calculated using a bicinechonic acid assay kit (Thermo Fisher Scientific). Proteins were resolved by SDS-PAGE and transferred to a methanol-activated polyvinylidene difluoride membrane. All primary and secondary antibodies were diluted in 5% donkey serum in TBS with 0.05% tween. Primary antibodies used included the following: anti-AKT2 (Cell Signaling, #3063), anti-pAKT2(S474) (Cell Signaling, #8959), anti-pGSK3β(S9) (Cell Signaling, #9323), anti-GSK3β (Cell Signaling, #9315), anti-IL1β (Abcam, ab34837), anti-IL-6 (Epitomics, 1957-1), anti-IRβ (Cell Signaling, #3025), anti-pIRβ(S994) (Thermo Fisher, purified custom antibody), anti-IRF3 (Santa Cruz Biotechnology, sc-9082), anti-pIRF3(S396) (Cell Signaling, #4947), anti-IRS1 (Cell Signaling, #3407), anti-p65 (Santa Cruz, sc-109), anti-pp65(S536) (Cell Signaling, #3031), anti-pTBK1(S172) (Cell Signaling, #5483), anti-TBK1 (Abcam, ab40676). Anti-β-actin (Sigma—Aldrich, A2066) and anti-GAPDH (Cell Signaling, #2118) were used as loading controls for all western blots shown. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody. Membranes were exposed with Clarity Western ECL Blotting Substrate (Bio-Rad) and visualized with the Odyssey Fc imager (LI-COR Biotechnology, Lincoln, NE).

2.5. Immunoprecipitation

Briefly, liver tissue lysates were prepared by lysis in TNET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris ph 8.0, 1% Triton X) plus protease and phosphatase inhibitor cocktails (Thermo Scientific) and 1 mM PMSF. Liver tissue lysate concentrations were assayed using a bicinicchonic acid assay and then equilibrated with lysis buffer. Lysates were pre-cleared by incubation with 20 μL of Protein A/G agarose beads (Thermo Scientific) for 90 min at 4 °C followed by centrifugation. Pre-cleared lysates (250 μg/sample) were then incubated with rabbit anti-TBK1 (Cell Signaling, #3031) or rabbit IgG control (Cell Signaling, #2729) and 30 μL of Protein A/G agarose beads and rotated overnight at 4 °C. Immunoprecipitates were washed three times in TNET buffer then heated to 95 °C for 5 min in standard SDS sample buffer. Samples were separated by SDS-PAGE followed by western blot analysis.

2.6. Histology and immunohistochemistry

Fat pads, livers, and pancreas tissues were excised and either frozen in liquid nitrogen and embedded in optimum cutting temperature compound (OCT, Tissue-Tek; EMS, Hatfield, PA) for frozen sections or fixed with 10% neutral buffered formalin solution overnight and embedded in paraffin for sectioning. Frozen liver sections (10 μm) were air-dried overnight and then fixed in 10% neutral buffered formalin for 10 min at room temperature. After brief rinsing with water and then 60% isopropanol, frozen liver sections were stained with oil red 0 for 15 min and counterstained with hematoxylin. Formalin-fixed paraffin embedded fat pads and pancreas tissues were cut into 5 μm sections. Paraffin sections were deparaffinized and rehydrated with xylene and serial dilutions of ethanol followed by H&E stain or antigen retrieval with 0.01 mol/L citric acid buffer (pH 6.0) for immunohistochemistry. Sections for immunohistochemical analysis were blocked with 20% aquablock and incubated with rabbit anti-CD11b/c (Novus Biologicals, NB110-40766) in blocking solution (5% BSA in TBS with 0.05% tween) at 4 °C overnight. Horseradish peroxidase–conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as a secondary antibody. Negative controls included omission of primary antibody. All slides were visualized with a Nikon Eclipse E600 microscope (Nikon, Melville, NY) and color images were captured using a Nikon Digital DX1200me camera and ACT-1 software. Images were analyzed using NIS Elements AR 2.3 Software (Nikon).

2.7. Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). Results are expressed as mean ± SEM. All data were analyzed by t-test or ANOVA. Significance was accepted at p < 0.05, with asterisks denoting p-value levels: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001.

3. RESULTS

3.1. HFD induces TBK1 expression and activity

Prolonged consumption of a HFD results in increased expression of IKKε and TBK1 in murine liver and WAT [14,15]. To confirm and extend these results, we evaluated IKKε and TBK1 expression in liver and subcutaneous WAT tissue collected from HFD-fed C57BL/6 mice. Consistent with earlier reports, TBK1 mRNA levels increased nearly 6- and 3-fold, respectively, in liver and WAT of HFD-fed animals (Figure 1A, B). Surprisingly, IKKε transcript levels were not elevated in either set of tissues post HFD, even though IL-6, which is known to be abundant in obese settings, was increased in liver and WAT, confirming the presence of metabolic inflammation in these HFD-fed animals [19,20]. In addition to confirming TBK1 induction at the protein level in liver tissues, we also observed an increase in TBK1 activity as seen by higher levels of pTBK1 and downstream TBK1 targets, pIRF3 and pp65 (Figure 1C). Interestingly, a previous study [21] reported that TBK1 interacts with insulin receptor β subunit (IRβ) in livers of obese rats. Concurrent with elevated TBK1 activity, we also detected a robust increase in IRβ-associated TBK1 by co-immunoprecipitation in liver lysates from HFD-fed mice compared to normal chow diet (ND) animals (Figure 1D). This heightened association could be indicative of a novel insulin-related function for TBK1.

3.2. Tbk1+/− mice are resistant to DIO

Upon observing the induction of TBK1 expression, activity and IRβ interaction on HFD feeding, we sought to resolve TBK1 contributions to metabolism that are distinct from its homolog, IKKε. We did this by utilizing Tbk1+/− mice [17]. To examine the outcome of metabolic stress in a Tbk1-deficient setting, we placed Tbk1+/− and Tbk1+/+ mice on HFD for 10 weeks. Weight measurements for each group were collected weekly and compared to age-matched ND-fed Tbk1+/− and Tbk1+/+ mice (Figure 2A). By 8 weeks of age (4 weeks in the study), we documented a 25% increase in total body weight in HFD-fed Tbk1+/− mice compared to ND. Similar to HFD-fed Ikkbe−/− mice and amlexanox-treated mice, Tbk1+/− mice showed only a modest increase in total body weight on HFD relative to ND-fed mice and overall maintained significantly lighter body weights than HFD-fed Tbk1+/− mice through the end of the study (Figure 2A, B). To confirm that these differences in body weight and fat mass are not due to Tbk1+/− mice eating less than Tbk1+/+ mice, we evaluated daily food intake relative to body weight. While no significant difference was observed, Tbk1+/− mice showed a minor increase in the ratio of HFD intake to body weight, eliminating the possibility of reduced consumption (Figure 2C). Additionally, there were no differences in body weight and length between ND-fed Tbk1+/− and Tbk1+/+ mice, indicating that the resistance to the DIO phenotype seen with Tbk1+/− mice is unique to this particular
Figure 1: Prolonged consumption of HFD induces TBK1 and promotes interaction with IR. mRNA expression of genes encoding TBK1, IKKe, and IL-6 in liver (A) and WAT (B) of C57BL/6 mice fed for 10 weeks with HFD or ND as indicated (n = 4 mice/group). Results are representative of mean ± SEM. Note: p = 0.06 for Tbk1 expression in WAT between ND and HFD. (C) Protein lysates from livers of ND- and HFD-fed C57BL/6 mice that were fasted overnight were immunoblotted with indicated antibodies and quantified. GAPDH was used as a loading control. (D) Liver lysates from ND- or HFD-fed mice were immunoprecipitated with an antibody against TBK1 or IgG, followed by immunoblotting and quantification of IRβ. Normal rabbit IgG was used as a control for specificity (IgG lane). Tissue lysates for immunoprecipitation were immunoblotted with antibodies against IRβ for input and GAPDH as a loading control. Statistical analysis by Student’s t-test. *p < 0.05, ****p < 0.0001.

Figure 2: Tbk1<sup>D/-</sup> mice are protected from HFD-induced weight gain. (A) Weekly body weights of Tbk1<sup>+/+</sup> and Tbk1<sup>D/-</sup> mice fed ND or HFD from 4 to 14 weeks of age (n = 7–10 mice/group). (B) Body weights of HFD-fed Tbk1<sup>+/+</sup> and Tbk1<sup>D/-</sup> mice at 4, 9 and 14 weeks of age. (C) Average daily food consumption of 14-week-old HFD-fed Tbk1<sup>+/+</sup> and Tbk1<sup>D/-</sup> mice was measured as gram of food per gram of mouse. (D) Circulating leptin levels were assayed in plasma from Tbk1<sup>+/+</sup> and Tbk1<sup>D/-</sup> mice as indicated. All mice are from 129S5 background. Results are representative of mean ± SEM. Statistical analysis by Student’s t-test. *p < 0.05, ***p < 0.001.
metabolic stress (Supplemental Fig. 1A,B). Next, we analyzed plasma leptin levels, which are usually elevated in obese individuals due to their higher percentage of body fat. We observed approximately a 40% reduction in circulating leptin in HFD Tbk1ΔΔ mice relative to Tbk1Δ/Δ mice, further highlighting the lower percentage of body fat in Tbk1ΔΔ mice (Figure 2D).

3.3. Tbk1ΔΔ mice are glucose and insulin tolerant on HFD

Given that the association of TBK1 with IRb was augmented on HFD and that loss of TBK1 protected mice from diet-induced weight gain, we examined the insulin-producing pancreatic beta cells in Tbk1Δ/Δ and Tbk1ΔΔ mice. Increased adipose tissue load resulting from excessive nutrient intake often leads to enhanced demands on pancreatic beta cells for insulin production. Pancreatic islets respond to the greater demand for insulin by increasing in number and in size. HFD-fed Tbk1ΔΔ mice were resistant to the increase in islet number and size that was observed in HFD-fed Tbk1Δ/Δ mice (Figure 3A–C). These results suggested that Tbk1ΔΔ mice are more tolerant of glucose and insulin on HFD compared to Tbk1Δ/Δ mice. OGTTs were performed in fasted (4 h) ND- and HFD-fed Tbk1Δ/Δ and Tbk1ΔΔ mice. As expected, significantly higher blood glucose measurements were observed in HFD-fed Tbk1Δ/Δ mice compared with ND-fed mice (Tbk1Δ/Δ and Tbk1ΔΔ) during the course of the OGTT. However, in contrast to HFD-fed Tbk1Δ/Δ mice, the high levels of blood insulin and glucose found in HFD-fed Tbk1Δ/Δ mice returned to basal values by 60 min and 120 min respectively, post oral gavage of glucose. These results indicate that TBK1 deficiency ameliorates diet-induced pancreatic islet stress and improves systemic glucose homeostasis, which is akin to the phenotype of previously reported lkb1−/− mice and amlexanox-treated animals (Figure 3D,E).

3.4. Loss of TBK1 kinase enhances insulin sensitivity on HFD

Munoz et al. [21] also reported that TBK1 can directly phosphorylate serine 994 (S994) of IRb in livers from obese rats. This particular serine site can be phosphorylated by the serine kinase, protein kinase C (PKC, isoforms β2 and δ) and has been implicated as an inhibitory phosphorylation site that blocks insulin receptor tyrosine kinase activity and subsequent downstream signaling [22,23]. Within metabolically diseased tissues from obese patients, there is a high level of proinflammatory cytokines that can drive activation of stress kinases, including Jun N-terminal kinase (JNK), IKKb and PKC [24,25]. These kinases are thought to respond to inflammation by constraining the activity of the insulin receptor directly or indirectly through IRSs via inhibitory phosphorylation, ultimately bringing insulin signaling down to baseline [26–28]. Given that TBK1 is a serine/threonine IKK (IκB kinase) family member that is responsive to inflammatory stimuli and is capable of phosphorylating the insulin receptor, we hypothesized that TBK1 negatively regulates insulin signaling through phosphorylation of S994 on IRb.

We examined the phosphorylation status of IRb (S994) in liver tissue from ND- and HFD-fed Tbk1Δ/Δ and Tbk1ΔΔ mice with an antibody specific for pIRb (S994). A specific pIRb (S994) signal was detectable only in livers from Tbk1ΔΔ mice in the HFD-fed group (Figure 4A). Furthermore, co-immunoprecipitation of TBK1 in liver lysates showed TBK1- IRb association after HFD feeding and also revealed a pIRb (S994) band exclusively in HFD Tbk1ΔΔ/ΔΔ livers (Figure 4B). These

Figure 3: HFD-fed Tbk1ΔΔ mice maintain glucose and insulin sensitivity. (A) Representative images of pancreata from adult Tbk1ΔΔ and Tbk1Δ/Δ mice after 10 weeks on ND or HFD. Black arrows point to pancreatic islets. Scale bar indicates 100 μm. (B) Number of islets counted per pancreas (4x section) in Tbk1ΔΔ and Tbk1Δ/Δ mice on ND or HFD (n = 4 mice/group). (C) Area of each islet from (B) measured in microns2. (D) Blood glucose and (E) plasma insulin measurements at indicated times after oral glucose injection for oral glucose tolerance tests in ND- or HFD-fed Tbk1ΔΔ and Tbk1Δ/Δ mice. For blood glucose, n = 4–10 mice/ND group and 8–12 mice/HFD group. For blood insulin, n ≥ 5 mice/group with exception at 15 min where n = 3 mice/group. All mice are from 129S6 background. Results are representative of mean ±/− SEM. Statistical analysis by Student’s t-test. *p < 0.05 and **p < 0.01 for HFD-fed Tbk1Δ/Δ mice compared to HFD-fed Tbk1ΔΔ mice. OGTT, oral glucose tolerance test.
results confirm that S994 of IRβ is phosphorylated in HFD-fed mouse livers and indicate that TBK1 is likely the upstream kinase responsible. Loss of this inhibitory signal in HFD-fed Tbk1+/− mice suggests that insulin signaling is intact in HFD-fed Tbk1+/− animals. Therefore, loss of TBK1 kinase activity in the context of a metabolic challenge results in the preservation of insulin sensitivity that is normally dampened as a result of HFD.

3.5. Tbk1+/− mice expend more energy through physical activity
If lack of functional TBK1 on HFD generates greater insulin responsiveness and subsequent glucose absorption in Tbk1 mutant mice, these animals are likely more efficient in storing and/or expending that energy. Total energy expenditure is composed of voluntary events, such as physical activity, and involuntary events, such as basal metabolic rate and thermogenesis. Thermogenesis was increased in lbkbe−/− mice and amlexanox-treated mice compared to control mice and was determined to be the primary difference contributing to their improved phenotype. The expression of thermogenic genes Ucp1, Ucp2, Pparg and Pparc1a were not significantly upregulated in HFD-fed Tbk1+/− mice compared to Tbk1+/− mice (Supplemental Fig. 2A,B). However, the thermogenic differences detected in lbkbe−/− mice and amlexanox-treated mice were at week 14 of HFD. Considering the 10-week duration of our HFD study, it is possible that differences in thermogenesis were not given sufficient time to manifest.

We evaluated alternative forms of energy expenditure by monitoring Tbk1+/− and Tbk1+/− mice in metabolic cages over a 72-hour period.
While there were no significant differences between ND-fed groups (Supplemental Fig. 3), HFD-fed Tbk1<sup>D/D</sup> mice were more active than Tbk1<sup>+</sup>/<sup>+</sup> mice as quantified by their increased movement (beam breaks) throughout the cages (Figure 5A). Accordingly, Tbk1<sup>D/D</sup> mice also had 22% higher oxygen consumption, 25% higher carbon dioxide production and 23% higher heat production, demonstrating greater energy expenditure overall (Figure 5B). Of note, the greatest differences in activity and energy expenditure took place during the dark cycles when the animals were awake. Since mice are nocturnal animals, any differences in resting metabolic rate between the two groups would be apparent during the light or daytime hours. Tbk1<sup>D/D</sup> mice had only a modest increase in movement and energy expenditure during light cycles, indicating that the enhanced nighttime physical activity observed in Tbk1<sup>D/D</sup> mice could account for their increased energy expenditure relative to Tbk1<sup>+</sup>/<sup>+</sup> mice.

3.6. HFD-fed Tbk1<sup>D/D</sup> mice maintain lipid homeostasis

Differences in fat mass and lipid accumulation were also examined. HFD induces adipocyte enlargement and proliferation in WAT. In subcutaneous fat pads collected from HFD-fed Tbk1<sup>+</sup>/<sup>+</sup> mice, we observed larger adipocytes compared to those collected from ND-fed Tbk1<sup>+</sup>/<sup>+</sup> mice (Figure 6A). While we did see larger adipocytes in fat pads from HFD-fed Tbk1<sup>D/D</sup> mice, overall, they contained a greater abundance of small adipocytes relative to HFD-fed Tbk1<sup>+</sup>/<sup>+</sup> fat tissue (Figure 6A,B). Additionally, cholesterol levels were measured from plasma of ND- and HFD-fed Tbk1<sup>+</sup>/<sup>+</sup> and Tbk1<sup>D/D</sup> mice. As expected, a prolonged fat diet led to a nearly 40% increase in circulating cholesterol in Tbk1<sup>+</sup>/<sup>+</sup> mice (Figure 6C). Yet, Tbk1<sup>D/D</sup> mice maintained cholesterol levels comparable to mice on ND. Furthermore, we performed oil red O staining on frozen liver sections to assess liver steatosis. Consistent with previous results, HFD Tbk1<sup>+</sup>/<sup>+</sup> mice displayed a striking level of hepatic lipid deposition while Tbk1<sup>D/D</sup> livers showed a 65% reduction in oil red O staining (Figure 6D,E). Despite prolonged consumption of a HFD, Tbk1<sup>D/D</sup>
mice preserve lipid homeostasis and maintain a consistently healthier phenotype.

3.7. Diet-induced inflammation is nominal in Tbk1 Δ/Δ mice

Chronic low-grade inflammation, also referred to as “meta-inflammation,” is a hallmark of metabolic syndrome and strongly associates with insulin resistance. It is characterized by a high degree of immune cell infiltration, primarily macrophages, and local cytokine production in metabolically diseased tissues. Meta-inflammation is thought to stem from pathological adipocyte tissue expansion in an obese state where the tissue becomes poorly oxygenated or hypoxic. Hypoxia leads to fibrosis and necrotic cell death in adipose tissue, which provokes local macrophage activation and/or polarization to an “M1” proinflammatory state. Inflammation is augmented by adipocytes and macrophages that generate proinflammatory cytokines to promote macrophage mobilization from bone marrow and local infiltration into adipose tissue and other metabolically diseased tissues [30]. In light of TBK1’s well-established role in mediating innate immunity and Tbk1 Δ/Δ mice being resistant to diet-induced lipogenesis, we suspected Tbk1 Δ/Δ mice would have limited meta-inflammation on HFD. In WAT stained for cd11b/c, we observed quantitatively fewer macrophages in HFD fed Tbk1 Δ/Δ mice relative to Tbk1 +/+ mice (Figure 7A). This difference in macrophage infiltration was confirmed in RNA isolated from WAT of HFD-fed Tbk1 Δ/Δ and Tbk1 +/+ mice (Figure 7B). Analysis of protein expression from liver lysates also revealed a reduction in IL-6 and IL-1β in HFD-fed Tbk1 Δ/Δ animals compared to Tbk1 +/+ (Figure 7C). Furthermore, WAT and liver protein lysates were analyzed for differences in cytokine expression by multiplex immunoassays. Cytokine levels showed little variance between ND-fed groups, which is consistent with the relatively mild immune phenotype of Tbk1 Δ/Δ animals (Supplemental Fig. 4A,B) [17]. Among the HFD-fed animals, Tbk1 Δ/Δ mice had less cytokine accumulation compared to Tbk1 +/+ mice and in particular, IL-6, IL-1β and TNFα levels were consistently lower in WAT and liver tissues from Tbk1 Δ/Δ mice (Figure 7D,E).

4. DISCUSSION

Numerous studies have demonstrated TBK1 as a key mediator in inflammatory and innate immune responses. However, recent evidence implicating TBK1 in the regulation of insulin receptor activity has emerged, leading us to investigate a novel metabolic function of this serine threonine kinase [21,31–34]. Our data corroborate previously reported upregulation of TBK1 expression on HFD and also show increased pIRF3, indicating heightened TBK1 kinase activity as well. In our DIO model, we confirmed the inhibitory interaction between TBK1 and IR] and demonstrate that insulin sensitivity is maintained under HFD conditions in the absence of functional TBK1, supporting a previously unappreciated function of TBK1 in regulating insulin receptor activity. Tbk1 Δ/Δ mice are resistant to DIO, and we suspect that this stems primarily from their enhanced insulin sensitivity, allowing for more efficient glucose uptake and utilization. Accordingly, these mice
exhibited greater energy expenditure primarily in the form of enhanced physical activity, which accounts for their leaner body composition. These results provide a mechanistic basis for the overall protective effect on the metabolism and general health of mice challenged with a HFD.

An important consideration with this study is that the mouse phenotype is due to loss in a Tbk1 gene) in HFD mice, thereby inhibiting its kinase activity and significantly reducing its overall expression in all cell types. Thus, it is unclear if the Tbk1ΔΔ mouse phenotype is due to Tbk1 loss in a specific subset or subsets of tissues or cell types. Interestingly, conditional deletion of Tbk1 in adipose tissue (Tbk1 ATKO) resulted in higher macrophage and proinflammatory cytokine gene expression on HFD [18]. In contrast to our model, HFD-fed Tbk1 ATKO mice were measured by Bio-Rad multiplex array (n = 4–8 mice/group for WAT, n = 8–12 mice/group for liver). All mice are from 129S5 background. Results are representative of mean ± SEM. Statistical analysis by Student’s t-test. *p < 0.05, **p < 0.01.

The relationship between inflammation and insulin resistance is complex and somewhat controversial. In particular, modulating the expression of certain inflammatory signaling molecules such as IKKβ, JNK, TNFα, IL-1R or IL-6 in different tissues or by different methods has led to inconsistent metabolic outcomes [36]. For example, liver specific JNK activation improved insulin sensitivity, yet activation of JNK in other tissues, including adipose tissue, skeletal muscle and brain, impaired insulin action, suggesting JNK regulation of insulin sensitivity is tissue specific [36–38]. Considering the well-established functions of Tbk1 in mediating inflammation and innate immunity, we cannot rule out the possibility that some of the healthier metabolic parameters seen in Tbk1ΔΔ mice could be due in part to reduced inflammation, especially given that Tbk1ΔΔ macrophages show only a partial response to LPS challenge [17]. While these results are consistent with the argument that inflammation leads to insulin resistance, the scope of this study limits the use of the inflammatory phenotype to function as an indicator of metabolic health in Tbk1ΔΔ mice. Regardless, our results strongly suggest that Tbk1 promotes metabolic dysfunction through negative regulation of insulin receptor signaling, thus aiding in insulin resistance.

This feature of Tbk1 is reminiscent of other kinases that have been previously shown to inhibit insulin signaling in response to cytokine activation. In particular, IKKβ, JNK, PKC, and S6K have each been shown to phosphorylate IRS proteins at unique sites that limit insulin-stimulated activity [26–28]. Accordingly, S6K1-deficient mice demonstrate a comparable phenotype to Tbk1ΔΔ mice on HFD with lower body weights and insulin hypersensitivity [39,40]. The opposition...
of insulin-stimulated anabolic processes common to all of these kinases is indicative of an evolutionary conserved role in reserved energy during times of inflammatory stress for resolving inflammation. In summary, we have characterized the phenotype of mice globally lacking catalytically active TBK1 under metabolic challenge, which, prior to this mouse, was impossible due to embryonic lethality in Tbk1−/− mice [16,17]. Overall, loss of TBK1 kinase activity benefited mice challenged with HFD. One way that TBK1 promotes metabolic syndrome progression is by directly impeding insulin receptor signaling, resulting in systemic insulin resistance and limited glucose absorption. While work from others has begun to elucidate the tissue specific effects of TBK1, it is unclear whether additional TBK1 substrates could be mediating these metabolic effects [18]. A recent study from Kumari and colleagues [41] revealed that the TBK1 substrate IRF3 transcriptionally drives adipose inflammation during periods of overnutrition. IRF3 was upregulated and more active in metabolic tissues from obese mice, matching our observations, while M3+/− mice were protected from DIO, exhibited reduced inflammation and showed improved insulin sensitivity on HFD. This finding helps elaborate the function of TBK1 under metabolic stress, but also highlights the need for further investigation of TBK1 targets in regulating metabolism.

Lastly, our results, in conjunction with other previous reports, imply that TBK1 inhibition may provide benefits to patients with metabolic syndrome and warrants further testing of pharmacological inhibitors of TBK1 in human trials [14,15,18,42,43]. The TBK1/IKKε inhibitor amlexanox just completed testing in clinical trials beginning with a safety trial of six patients in which no serious adverse events were reported from drug treatment (NCT01842282) [44]. The safety trial was followed by a randomized, double-blind, placebo-controlled trial of 42 obese patients with type 2 diabetes or nonalcoholic fatty liver disease who received amlexanox or placebo for 12 weeks (NCT01975935). A subset of patients constituting one-third of the participants in the blinded study responded with a clinically significant reduction in blood glucose. Interestingly, molecular analysis of fat tissue biopsies collected from patients at the beginning of the study revealed greater inflammation in the responder group compared to non-responders. Though it is unclear whether this high level of inflammation in the responder group showed any sort of resolution by the end of the study, further development and confirmation of this inflammatory gene signature could lead to its utilization as a predictive tool for patient response to amlexanox or potentially other TBK1 inhibitors. Future studies are needed with larger patient groups to validate the efficacy of amlexanox, determine the appropriate dosage and dosing schedule and evaluate whether amlexanox treatment has long-term benefits.

AUTHOR CONTRIBUTIONS

VHC, PES and RAB conceived and designed the study. VHC, ENA and KWW acquired data and performed analysis and interpretation of data. VHC wrote the manuscript. PES and RAB reviewed and revised the manuscript. RAB supervised the study.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.06.007.

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