Thromboxane synthase deficiency improves insulin action and attenuates adipose tissue fibrosis

Authors: Xia Lei1,†, Qing Li1,2,†, Susana Rodriguez1, Stefanie Y. Tan1, Marcus M. Seldin1, John C. McLenithan3, Weiping Jia2, and G. William Wong1,*

Affiliation:

1. Department of Physiology and Center for Metabolism and Obesity Research, The Johns Hopkins University School of Medicine, Baltimore, MD 21205;

2. Department of Endocrinology and Metabolism, Shanghai Jiao Tong University affiliated Sixth People’s Hospital, Shanghai Diabetes Institute, Shanghai Clinical Center of Diabetes, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai 200233, China;

3. Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201

AUTHOR CONTRIBUTION

X.L., Q.L., and G.W.W. contributed to the experimental design; X.L., Q.L., S.R., S.Y.T., and M.M.S. performed the experiments; J.C.M. and W.J. contributed to analytic tools; Q.L., X.L., and G.W.W. analyzed and interpreted the data; Q.L., X.L. and G.W.W. wrote the paper.

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*Corresponding author: G. William Wong, E-mail: gwwong@jhmi.edu, Department of Physiology and the Center for Metabolism and Obesity Research, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Tel: 410-502-4862
Fax: 410-614-8033

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ABSTRACT

Thromboxane A2, an arachidonic acid-derived eicosanoid generated by thromboxane synthase (TBXAS), plays critical roles in hemostasis and inflammation. However, the contribution of thromboxane A2 to obesity-linked metabolic dysfunction remains incompletely understood. Here, we used in vitro and mouse models to better define the role of TBXAS in metabolic homeostasis. We found that adipose expression of Tbxas and thromboxane A2 receptor (Tbxa2r) was significantly upregulated in genetic and dietary mouse models of obesity and diabetes. Expression of Tbxas and Tbxa2r was detected in adipose stromal cells, including macrophages. Further, stimulation of macrophages with interferon-γ or resistin—factors known to be upregulated in obesity—induced Tbxas and Tbxa2r expression. Mice lacking Tbxas had similar weight gain, food intake, and energy expenditure. Loss of Tbxas, however, markedly enhanced insulin sensitivity in mice fed a low-fat diet. Improvement in glucose homeostasis was correlated with the upregulated expression of multiple secreted metabolic regulators (Ctrp3, Ctrp9, and Ctrp12) in the visceral fat depot. Following a challenge with a high-fat diet, Tbxas deficiency led to attenuated adipose tissue fibrosis and reduced circulating IL-6 levels without affecting adipose tissue macrophages; these changes, however, were not sufficient to improve whole-body insulin action. Together, our results highlight a novel, diet-dependent, role for thromboxane A2 in modulating peripheral tissue insulin sensitivity and adipose tissue fibrosis.

Key words
Adipose tissue, eicosanoid, thromboxane, fibrosis, obesity, diabetes, CTRP
INTRODUCTION

Arachidonic acids released from the plasma membrane by phospholipases can be converted to eicosanoids, a class of lipids that include the prostaglandins, thromboxane, and leukotrienes (28, 50). These signaling lipids play pivotal and pleotropic roles in wide-ranging physiological processes, including development, tissue homeostasis, immunity, inflammation, and reproduction (15). Dysregulated production of eicosanoids underlies the pathogenesis of many diseases, such as allergic inflammation, atherosclerosis, and cardiovascular disease (36, 43, 51, 56). Consequently, enzymes involved in the synthesis of eicosanoids are major targets of pharmaceutical drugs, including the widely prescribed nonsteroidal anti-inflammatory drugs (NSAID) (6, 44).

While leukotrienes are generated through the lipoxygenase pathway, the prostanoids (prostaglandins and thromboxane) are generated through the cyclooxygenase (COX) pathway (15). COX-1 and COX-2 are the two major enzyme isoforms that convert arachidonic acid to prostaglandin H2 (PGH2), which in turn serves as a substrate for the synthesis of other prostaglandins and thromboxane (50). While COX-1 is widely and constitutively expressed, COX-2 is the inducible isoform with a more restricted tissue distribution (12); both of these enzymes are targeted by NSAID such as aspirin (46, 59).

Recent studies using cyclooxygenase-2 (Cox-2)-deficient mice showed that arachidonic acid-derived eicosanoids, such as 15d-PGJ2, are important for adipocyte differentiation in vivo (16). Loss of COX-2 reduces body weight in aged (>8 months old) mice and attenuates adipose tissue inflammation (16). The COX-2 enzyme, normally found at very low levels in immune cells, is induced by inflammatory stimuli. Through enzymatic and non-enzymatic pathways, COX-2-derived PGH2 is further converted to other prostanoids with distinct functions, including PGD2, PGE2, PGF2α, PGJ2, and thromboxane A2 (60). Thus, in Cox-2-deficient mice, synthesis of multiple types of prostanoids may be affected. As such, the contribution of specific eicosanoids to obesity-linked metabolic dysfunctions remains incompletely defined.

Thromboxane synthase (TBXAS) catalyzes the conversion of prostaglandin H2 to thromboxane A2 (11, 22, 33, 49). Due to its short half-life (~30 s) and rapid conversion to the inactive form (thromboxane B2), thromboxane A2 acts locally in an autocrine or paracrine fashion (47). By binding to the G protein-coupled thromboxane A2 receptor (TBXA2R) on vascular endothelial and smooth muscle cells, thromboxane A2 regulates hemostasis by modulating platelet aggregation and vasoconstriction (24, 47). Indeed, targeted deletion of Thxas or Tbxar2 in mice results in hemostasis defects (54, 68). While platelets produce thromboxane in clotting blood, the major source of thromboxane in inflammation is derived from immune cells (23, 57). Mice lacking Tbxas2R have reduced inflammatory response to tissue injury (55). Excess thromboxane A2 has also been linked to atherosclerosis (27, 31), glomerulonephritis (42), and hypertension (8, 13). In humans, serum levels of thromboxane B2 (a stable metabolite of thromboxane A2) are found to be significantly elevated in obese subjects relative to lean individuals (18); paradoxically, in morbidly obese (average BMI of 49) but insulin-sensitive subjects, serum thromboxane B2 levels are found to be lower than in healthy lean individuals (18). In the context of diabetes, both type 1 and type 2 diabetic individuals have higher serum thromboxane B2 levels (41); the production of thromboxane B2 is also correlated with fasting plasma glucose and hemoglobin A1c (HbA1c). In studies involving obese females with elevated thromboxane levels, weight loss or pioglitazone treatments also result in significant reduction of urinary thromboxane levels (4, 9). While the role of thromboxane in obesity-linked metabolic dysregulation has not been examined, the correlative studies in humans prompted us to further explore its metabolic function in the context of obesity and diabetes.

One of the hallmarks of obesity is the striking recruitment of proinflammatory macrophages into adipose tissue and the ensuing inflammatory sequela (21, 25, 64, 67). Elevated expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 by infiltrated macrophages contributes to chronic low-grade inflammation and adipose tissue insulin resistance and dysfunction (25). Given that thromboxane A2 is produced by activated macrophages (7, 57) and has potent proinflammatory activity (56), this study aimed to uncover the role of this lipid mediator in obesity-linked metabolic dysfunction using a whole-body knockout mouse model devoid of the key terminal enzyme (TBXAS) that synthesizes thromboxane A2.
Glossary

Col  collagen
DIO  diet-induced obesity
G6Pc  Glucose-6-phosphatase
HbA1c  Hemoglobin A1c
HIF1-α  hypoxia inducible factor 1 alpha
HFD  high-fat diet
IFN-γ  interferon gamma
IL-1β  interleukin-1 beta
IL-6  interleukin-6
KO  knockout
LFD  low-fat diet
MCP-1  monocyte chemotactic protein 1
MMP-12  matrix metalloprotease 12
NEFA  non-esterified free fatty acid
NOS2  nitric oxide synthase 2
PCK1  Phosphoenolpyruvate carboxykinase
TNF-α  tumor necrosis factor alpha
TBXAS  thromboxane synthase
TBXA2r  thromboxane A2 receptor
WAT  white adipose tissue
eWAT  epididymal white adipose tissue
iWAT  inguinal white adipose tissue
WT  wild-type
15d-PGJ2  15-deoxy-Δ(12,14)-prostaglandin J2
EXPERIMENTAL PROCEDURES

Mice
All animal experiments were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Male wild-type (WT), leptin-deficient obese (ob/ob), and Tbxas-/- mice (all on a C57BL/6J genetic background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were allowed to acclimate to the animal facility for at least 1 week. Tbxas-/- knock-out (KO) mice and Tbxas+/- WT littermate controls were generated by crossing Tbxas+/- heterozygous breeding pairs. The genotypes of Tbxas WT, heterozygous, and KO mice were confirmed by PCR (68). Age- and sex-matched mice of both sexes were used in all studies unless otherwise stated. Male and female Tbxas WT and KO mice from 4 to 24 weeks old were used. Laboratory mice consumed standard chow diet (no. 5001, Lab Diet, St. Louis, MO), had free access to water, and were housed in polycarbonate cages on a 12-h light/dark photocycle. Four-week-old C57BL/6J male mice or Tbxas WT and KO mice were fed a high-fat diet (HFD) (60 kcal% derived from fat; D12492; Research Diets, New Brunswick, NJ) or a matched control low-fat diet (LFD) (10 kcal% derived from fat; D12450B; Research Diets) for 12-14 weeks. Body weights of Tbxas WT and KO mice were measured weekly.

To assess direct insulin action in vivo, a subset of WT and KO mice from each diet group was injected with saline control or insulin (1 U/kg of body weight) via intraperitoneal (i.p.) route 15 min before euthanization. Epididymal white adipose tissue (eWAT), liver, and skeletal muscle were quickly removed for RNA and protein extraction or for histological study. Tissues were collected, weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Blood samples were collected for serum analysis. The fed, fasted, re-fed experiments in WT C57BL/6J male mice were performed as previously described (39).

Isolation of primary adipocytes and stromal vascular cells from adipose tissue
Twenty-week-old C57BL/6J WT male mice, fed a standard laboratory chow, were used to obtain primary adipocytes and stromal vascular cells (stromal vascular fraction; SVF) from epididymal white adipose tissue as described previously (65).

Cell culture
Mouse 3T3-L1 fibroblasts were cultured and differentiated into adipocytes as previously described (63). Mouse RAW264.7 macrophage cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and antibiotics. RAW264.7 macrophages or differentiated 3T3-L1 adipocytes were stimulated with PBS control, IL-1β (2 ng/mL), IL-6 (2 ng/mL), TNF-α (2 ng/mL), IFN-γ (50 ng/mL), or resistin (100 ng/mL) for 6 h. Total RNA was isolated, reverse-transcribed, and subjected to quantitative real-time PCR (qRT-PCR) analysis of Tbxas and Tbx2a2r expression.

RNA isolation and real-time PCR analysis
Total RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA) and reverse-transcribed using GoScript™ Reverse Transcriptase (Promega, Madison, WI). Ten nanograms of cDNA from each sample were analyzed by quantitative real-time PCR on a CFX Connect system (Bio-Rad Laboratories, Hercules, CA). Samples were analyzed in 25-µL reactions using SYBR® Green PCR master mix per manufacturer’s instructions (Applied Biosystems/Life Technologies). Expression was normalized to 18S rRNA for mouse samples. Primer sequences are listed in Table 2. The primer sequences used to assess Ctrp1, Ctrp3, Ctrp9, and Ctrp12 gene expression in adipose tissue were previously described (62, 65, 66).

Cell lysate preparation and western blotting
Frozen mouse tissues were thawed and minced in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol) supplemented with PhosSTOP phosphatase inhibitor cocktail (Roche, Basel, Switzerland) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Tissues were further disrupted and homogenized with a Benchmark D1000 tissue homogenizer. Tissue lysates were centrifuged at 10,000 x g for 20 min at 4°C to remove insoluble materials. Protein concentration was determined using the Bradford protein assay (Sigma-Aldrich, St. Louis, MO). Twenty micrograms of protein lysate were separated...
on 10% Bis-Tris NuPAGE gels (Invitrogen). Western blots and quantifications were carried out as previously described (62). Phospho-AKT (Thr-308) and AKT antibodies were obtained from Cell Signaling Technology.

**Histology**

Formalin-fixed, paraffin-embedded white adipose tissue sections were stained with hemotoxylin and eosin (HE) at the Pathology Core facility at Johns Hopkins University School of Medicine. Images were captured with a Zeiss Axioplan upright microscope with a Zeiss Axioacam color CCD camera (Carl Zeiss Microscopy, Thornwood, NY). Masson's trichrome staining (AML Laboratories, Baltimore, MD) was performed on paraffin-embedded tissue sections to visualize collagen deposition in the extracellular matrices of adipose tissue.

**Glucose and insulin tolerance test**

Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as previously described (39) on Tbxas WT and KO mice fed a HFD or LFD for 10-20 weeks. For GTT, mice were fasted overnight before i.p. injection of 1 g glucose/kg body weight (BW). Blood was collected via tail bleed before and after injection and glucose concentrations were measured using a glucometer (BD Biosciences, San Jose, CA) at the indicated time point. For ITT, food was removed 2 h before i.p. injection with 1 U insulin/kg BW. Blood glucose concentrations were measured at the indicated time points.

**Body composition analysis**

Body composition of Tbxas WT and KO mice was measured using a whole-body EchoMRI NMR instrument (Echo Medical Systems, Waco, TX) housed at the Molecular and Comparative Pathobiology Phenotyping Core facility at the Johns Hopkins University School of Medicine. EcoMRI analyses measured fat mass, lean mass, and water content.

**Indirect calorimetry**

Tbxas WT and KO mice (n=10 per group) were used for simultaneous assessments of daily body weight change, energy intake (corrected for spillage), and whole-body metabolic profile in an open-flow indirect calorimeter (CLAMS, Columbus Instruments, Columbus, OH). Data were collected for 3 days to confirm acclimation to the calorimetry chambers (stable body weights and food intakes), and data from day 4 in CLAMS were analyzed. Rates of oxygen consumption (VO2, mL/kg lean mass/h), carbon dioxide production (VCO2), physical activity, and food intake were measured as previously described (37, 38). Average metabolic values were calculated per subject and averaged across subjects for statistical analysis.

**Blood chemistry analysis**

Tail vein blood samples were collected in the morning (10:00 a.m.) from mice that were fasted for 15 h during the dark cycle (7:00 p.m.–10:00 a.m.). Samples were allowed to clot on ice, and then centrifuged for 10 min at 10,000 × g. Serum samples were stored at -80°C. Serum insulin, leptin, adiponectin, IL-6, IL-10, TNF-α (Millipore, Billerica, MA), and MCP-1 (R&D Systems, Minneapolis, MN) levels were measured by ELISA according to manufacturer’s instructions. Serum triglycerides and non-esterified free fatty acids were measured using Wako kit. Serum thromboxane B2 (a stable metabolite of thromboxane A2) was measured using an ELISA kit (CSB-E08048m, CUSABIO) according to the manufacturer’s instructions.

**Statistical analysis**

Kruskal-Wallis test with Dunn’s Multiple Comparison post hoc test, and Student’s t-tests were used to determine significant differences between groups. Statistical analyses were performed with GraphPad Prism (GraphPad Prism, San Diego, CA, USA), and values were considered significant at \( P<0.05 \). All data are presented as mean ± S.E.
RESULTS

Metabolic perturbations alter Tbxas and Tbxa2r expression in mice

To establish the physiological relevance of thromboxane A2 to metabolism, we first examined whether changes in metabolic states affects the expression of Tbxas and Tbxa2r. In a genetic model of severe obesity, i.e., leptin-deficient ob/ob mice, Tbxas and Tbxa2r expression was upregulated in epididymal white adipose tissue (Fig. 1A,B). In a diet-induced obese (DIO) mouse model, more akin to the common form of human obesity, the expression of Tbxas was upregulated in visceral (epididymal), but not subcutaneous (inguinal), white adipose tissue (Fig. 1C,E). The expression of thromboxane receptor (Tbxa2r), however, was similarly upregulated in both visceral (epididymal) and subcutaneous (inguinal) white adipose tissue of DIO mice (Fig. 1D,F) mouse models. In contrast to adipose tissue, the expression of Tbxa2r in liver and skeletal muscle was not different between mice fed an LFD or HFD (Fig. 1G). As expected based on their distribution in platelets and immune cells (22, 57), most Tbxas and Tbxa2r transcripts found in adipose tissue were produced by stromal cells [referred to as the stromal vascular fraction (SVF)] rather than mature adipocytes (Fig. 1H,I). Interestingly, acute metabolic changes, such as an overnight fast, also induced Tbxas and Tbxa2r expression in subcutaneous (inguinal), but not visceral (epididymal), fat depots (Fig. 1J,K).

Proinflammatory cytokines up-regulate Tbxas and Tbxa2r expression in macrophages in vitro

In the obese state, local expression of proinflammatory cytokines is upregulated in adipose tissue (21, 25, 64). We examined if the same factors that promote inflammation and insulin resistance would alter the expression of Tbxas and Tbxa2r in macrophages, which are known to infiltrate adipose tissue in the obese state (21, 64, 67) and serve as a major source of thromboxane A2 in inflammation (56). RAW264.7 macrophages stimulated with recombinant IFN-γ or resistin for 6 h significantly upregulated expression of both Tbxas and Tbxa2r (Fig. 1L,M). No effects were seen when cells were treated with IL-1β, IL-6, and TNF-α. Consistent with macrophages, rather than adipocytes, being a major producer of thromboxane A2 in adipose tissue, expression of Tbxas and Tbxa2r was low and unchanged in 3T3-L1 adipocytes upon differentiation (data not shown). Tbxas and Tbxa2r expression in differentiated adipocytes was also unaffected by treatment with IL-1β, IL-6, TNF-α, IFN-γ, or resistin (data not shown).

Impact of Tbxas deficiency on body weight, adiposity, and adipose tissue histology of mice fed a low-fat diet

A Tbxas-deficient mouse model (68) was used to determine the contribution of Tbxas to local (adipose tissue) and systemic energy balance in normal or pathophysiological contexts of diet-induced obesity. Although thromboxane A2 is the primary physiological agonist for TBXA2r (24), other molecules such as PGH2, isoprostanes, and hydroxyeicosatetraenoic acids are also potent agonists for TBXA2r (3, 17); further, epoxyeicosatrienoic acids can act as endogenous antagonists of TBXA2r (5). Based on this consideration, we chose to use Tbxas, rather than Tbxa2r, KO mice to address the contribution of thromboxane A2 to metabolic homeostasis. Four-week-old Tbxas WT and KO mice were fed an HFD or LFD for 20 weeks. We observed no differences in body weight gain over time between WT or KO mice fed an LFD (Fig. 2A). Body composition analysis using NMR indicated no differences in fat or lean mass between the two groups of mice (Fig. 2B). Visceral (epididymal) and subcutaneous (inguinal) white adipose tissue histology was not different between WT and KO animals (Fig. 2C). Although the gross morphology of adipose tissue looked comparable, we examined the expression of fibrotic (Col1, Col3, Col6), adipose macrophage (F4/80 and Cd11c), and pro-inflammatory macrophage M1 (Nos2) marker gene expression in both the visceral and subcutaneous fat depot of Tbxas WT and KO mice (Fig. 2D-I) and did not observe significant differences between the two groups.

Tbxas deficiency improves peripheral tissue insulin action in mice fed a low-fat diet

We next performed indirect calorimetry analysis on LFD-fed mice. No differences were observed in food intake, rate of oxygen consumption (VO2), energy expenditure, or physical activity levels between WT and KO mice (Fig. 3A-D). Despite similar body weight and adiposity, KO mice had enhanced insulin sensitivity, as indicated by a much greater rate of glucose disposal in the peripheral tissue (Fig. 3E,F); the magnitude of insulin secretion during GTT was not significantly different between the two groups (data not shown).
Improved insulin action in the Tbxas KO mice was further confirmed by insulin tolerance tests (Fig. 3G,H). Next, we injected insulin into a separate cohort of LFD-fed WT and KO animals to directly assess the activation of insulin signaling in three major metabolic tissues. Before insulin administration, we observed an ~2-fold increase in basal AKT phosphorylation (a metric of insulin signaling) in the adipose tissue but not liver or skeletal muscle of KO mice relative to WT controls (data not shown). At 15 min after insulin administration, we observed a robust insulin-stimulated AKT phosphorylation in all three tissues in both Tbxas WT and KO mice. While the levels of AKT phosphorylation were higher in the KO mice (after normalization to total AKT), the difference fell short of being statistically significant and this may be attributed to a small sample size (n=3) and the length of insulin stimulation (15 min).

Enhanced insulin sensitivity in LFD-fed KO mice, as judged by glucose and insulin tolerance tests, appeared to be independent of serum TG, adiponectin, leptin, and IL-6 levels (Fig. 4A-E). Only serum NEFA levels were modestly higher in KO mice compared to WT controls (Fig. 5B). Examination of hepatic gluconeogenic gene (G6Pc and Pck1) expression revealed no differences between WT and KO mice (Fig. 4F). Expression of insulin-responsive glucose transporter 4 (Glut4) gene was higher in the visceral (epididymal) adipose tissue of Tbxas KO mice (Fig. 4G). We have previously shown that secreted proteins of the C1q family, the C1q/TNF-related proteins (CTRPs), play important roles in regulating insulin sensitivity, and glucose and lipid metabolism in vivo (37-40, 61, 62). We therefore also examined the expression of Ctrp in adipose tissue of Tbxas WT and KO mice; we focused our analysis on Ctrp1, Ctrp3, Ctrp9, and Ctrp12 based on their previously described in vivo metabolic functions (37-40, 61, 62). Three of the Ctrp transcripts were upregulated in visceral (epididymal), but not subcutaneous (inguinal), white adipose tissue of Tbxas KO mice (Fig. 4H).

**Tbxas deficiency attenuates adipose tissue fibrosis in HFD-fed mice**

We next subjected Tbxas WT and KO mice to metabolic stress induced by high-fat feeding. When Tbxas KO mice were challenged with an HFD for a period of 16 weeks, we observed no differences in body weight or fat and lean mass between WT and KO mice (Fig. 5A,B). As expected from TBXAS-deficient mice, serum thromboxane B2 (TXB2), a stable metabolite of thromboxane A2, was largely, if not completely, abolished when compared to WT controls (Fig. 5C). The lowest assay detection limit for TXB2 in mouse serum is 2.4 pg/mL; we therefore could not distinguish the apparent residual TBX2 seen in the Tbxas KO mouse sera (~2.8 pg/mL) from background signals. As with the LFD-fed groups, we also performed indirect calorimetry analysis on the HFD-fed animals. Tbxas KO mice fed an HFD also had similar food intake, metabolic rate (VO2), energy expenditure, and physical activity levels when compared to WT controls (Fig. 5D-G).

Since macrophage-derived thromboxane A2 acts locally due to its short half-life (20, 47), we further examined the local milieu of white adipose tissue. The extracellular matrix (ECM) plays an important role in adipose tissue expansion in response to excess caloric intake (26, 52). Diet-induced obesity also results in adipose tissue hypoxia and fibrosis (19). We therefore examined the histology and expression of multiple ECM markers in both the visceral (epididymal, eWAT) and subcutaneous (inguinal, iWAT) fat depots of Tbxas WT and KO mice. Histological analysis of adipose tissue sections revealed differences between Tbxas WT and KO mice (Fig. 6A,B). KO animals had better preservation of adipose tissue architecture, with reduced numbers of stromal vascular cells interspersed among adipocytes (Fig. 6A); majority of the non-adipocytic cells are infiltrated immune cells, especially macrophages, as has been previously shown (64, 67). Adipose histology also demonstrated decreased deposition of fibrotic collagens (indicated by Masson’s trichrome stain) in comparison with WT animals (Fig. 6B). Trichrome staining specifically highlights the presence of fibrillar collagens I and III, yielding a blue stain. Whereas trichrome staining of HFD-fed WT mice contained pronounced trichrome-positive “streaks” interspersed among the adipocytes, adipose tissue from KO mice revealed only thin collagen sheets surrounding each adipocyte. In support of the histology data, expression of adipose fibrosis-promoting collagen genes Col1 and Col3 was significantly reduced in eWAT (Fig. 6C,D), whereas the adipose expression of Col6 (Fig. 6E) and Hif1-α (not shown) did not differ between the two groups of mice.

Diet-induced obesity is known to result in macrophage infiltration into adipose compartment (64, 67). We therefore determined whether there is any difference in adipose macrophages between Tbxas WT and KO
animals. Expression of macrophage-specific markers F4/80 and Cd11c was not significantly different in the adipose tissue of WT and KO mice (Fig. 6F,G). The expression of Nos2, a marker of the proinflammatory M1 macrophage subtype, was also not different between WT and KO mice (Fig. 6H); nor were there differences in the adipose expression of Tnf-α (data not shown). Thus, loss of Tbxas in mice had no apparent effect on the inflammatory state of adipose tissue in mice fed an HFD. In HFD-fed mice, we also examined the expression of Ctrp1, Ctrp3, Ctrp9, and Ctrp12 in epididymal white adipose tissue and did not observe any differences between WT and KO mice (data not shown).

To examine the effects of Tbxas deficiency on glucose metabolism, we measured fasting (5 h fast) blood glucose concentrations every other week in Tbxas WT and KO mice. No differences were seen between the two groups over a 14-week period (data not shown). In contrast to KO mice fed an LFD, we observed no differences in glucose and insulin tolerance tests (GTT) between Tbxas WT and KO mice fed an HFD (Fig. 7A-D), nor any differences in serum TG, NEFA, adiponectin, IL-10, MCP-1, or TNF-α (Fig. 7E-G, J-L). Interestingly, serum leptin levels were higher and IL-6 levels were lower in the KO group relative to WT controls (Fig. 7H,I). Thus, enhanced insulin sensitivity seen in the LFD-fed Tbxas KO animals was abrogated when mice were subjected to high-fat feeding.

DISCUSSION

In the present study we sought to uncover the role of thromboxane A2 in a dietary model of obesity. We provide evidence that thromboxane A2, generated by TBXAS, contributes to whole-body insulin sensitivity and obesity-linked adipose tissue fibrosis. Although loss of Tbxas had no impact on food intake, weight gain, adiposity, or energy expenditure, it enhanced insulin action in the peripheral tissue of mice fed an LFD. When challenged with an HFD, TBXAS deficiency helped attenuate adipose tissue fibrosis without any apparent effect on infiltrating macrophages. The expression of Tbxas and Tbx2r in white adipose tissue was markedly increased in genetic and dietary mouse models of obesity. Our mouse expression data on Tbx2r are consistent with recent human studies showing that serum thromboxane B2 levels are elevated in obese and/or diabetic subjects (18, 41) and that weight reduction from decreased caloric intake or pioglitazone treatment leads to reduced urinary thromboxane B2 levels (4, 9). Further, our genetic loss-of-function studies in Tbxas KO mice provide functional evidence to support a role for thromboxane in modulating peripheral tissue insulin sensitivity and glucose homeostasis. Thus, our findings underscore the clinical relevance of thromboxane A2 to metabolic dysregulation in humans.

Leptin and ghrelin are among the many circulating hormones whose levels can be altered by fasting and refeeding. Plasma leptin levels are very low in fasted mice and increased upon refeeding (1). When leptin levels are low, as in the fasted state, the expression of both Tbxas and Tbx2r in white adipose tissue is increased. Consistent with this, in leptin-deficient ob/ob mice, we observed a significant increase in the expression of both Tbxas and Tbx2r. While leptin levels are inversely correlated with Tbxas and Tbx2r expression, we do not know whether leptin directly regulates the expression of these two genes. The leptin-deficient mice are hyperphagic and morbidly obese; thus, obesity may, in part, be responsible for the upregulated expression of Tbxas and Tbx2r. In support of this, we also observed an increase in Tbxas and Tbx2r expression in diet-induced obese mice.

In the obese state, large numbers of macrophages infiltrate adipose tissue (21, 25, 64, 67). Activated macrophages (30, 35) and, to a lesser extent, adipocytes produce and secrete proinflammatory cytokines. These factors, in turn, create a state of chronic low-grade inflammation within the adipose compartment, leading to impaired insulin action and adipocyte dysfunction (21, 25, 64, 67). Some of these proinflammatory cytokines (e.g., resistin) also induce Tbxas expression in macrophages. Recent studies suggest that macrophage populations are heterogeneous and consist of multiple subtypes (29, 30, 32). M1-type macrophages promote inflammation, while M2-type macrophages play an anti-inflammatory role through the cytokines they secrete (32, 34). In the context of obesity, M1-type macrophages are recruited into the adipose compartment (30). However, adipose tissue function and systemic insulin sensitivity can be significantly improved when macrophages are polarized toward the M2-type phenotype (35). Adipocytes secrete many adipokines (e.g.,
leptin, adiponectin, resistin) that help maintain energy homeostasis (45). When adipocyte function is compromised by chronic local inflammation due to DIO, whole-body metabolism is affected (25, 34). Since TBXAS-generated thromboxane A2 has proinflammatory activity (56) and acts locally, it may exacerbate the inflammatory state in adipose tissues. However, in Tbxas KO mice fed an LFD or HFD, we observed no differences in the inflammatory state of adipose tissue compared to WT controls.

Since adipose tissue specializes in triglyceride storage, its coordinated expansion in response to excess caloric intake is crucial to maintain lipid homeostasis and prevent aberrant lipid deposition in non-adipose tissues (e.g., liver and skeletal muscle) that can promote insulin resistance (58). HFD-induced fibrosis in the adipose compartment compromises adipose tissue expandability and hence its capacity to handle excess dietary lipids (26, 53). In HFD-fed Tbxas KO mice, expression of fibrotic collagens (Coll1 and Col3) is decreased, leading to reduced collagen deposition in the ECM. Reduced adipose tissue fibrosis, however, was not sufficient to improve whole-body glucose and insulin tolerance in HFD-fed Tbxas KO mice compared to WT controls.

Because COX-derived PGH2 can be converted to different prostaglandins and thromboxane, we compared and contrasted our present findings with two recent studies, one of which involved the use of Cox-2 KO mice (16) and another involved transplanting Cox-1+/+ and Cox-1−/− bone marrow cells into lethally irradiated WT recipient mice to reconstitute the hematopoietic compartment (48). The use of whole-body Cox-2 KO mice demonstrates the importance of eicosanoids in adipocyte differentiation in vivo (16). In chow-fed Cox-2 KO mice, reduced body weight and fat mass is attributed, in part, to reduced adipogenesis resulting from decreased production of 15-deoxy-Δ(12,14)-PGJ2, an activating ligand for PPAR-γ that plays a critical transcriptional role in orchestrating the adipogenic program (14). The levels of other prostaglandins, including PGD2, PGE2, PGA2, and 6-keto-PGF1α, are not different in the adipose tissue explants of Cox-2 WT and KO mice (16). We do not know if thromboxane A2 was affected in Cox-2-deficient mice (16). The striking differences in body weight between Cox-2 WT and KO mice emerged when these animals aged (>8 months); this was attributed, in part, to increased energy metabolism without changes in food intake of Cox-2 KO animals (16). In contrast to the Cox-2 KO animals, Tbxas WT and KO mice fed an LFD or HFD did not differ in food intake, body weight, or adiposity, although the study duration described here was less than 4 months.

Despite the striking differences in body weight and adiposity, there appears to be no differences in non-fasting blood glucose, triglycerides, and cholesterol levels between Cox-2 WT and KO mice (16). Since glucose and insulin tolerance tests were not performed, we do not know if the Cox-2 KO mice have improved glucose homeostasis. In contrast to the Cox-2 KO mice, loss of TBXAS significantly improved insulin sensitivity in mice fed an LFD compared to WT controls, as judged by both glucose and insulin tolerance tests. Since circulating levels of leptin, adiponectin, or IL-6 were not different between Tbxas WT and KO mice, improvements in insulin action in Tbxas KO mice were independent of these adipokines known to modulate whole-body insulin sensitivity (45). Hepatic expression of gluconeogenic genes (G6Pc and Pck1) was not different between Tbxas WT and KO mice; hence, improved glucose homeostasis was likely not due to changes in hepatic glucose output. We did, however, observe a higher expression of insulin-responsive glucose transporter Glut4 in the adipose tissue of KO mice. Further, we observed a significant increase in the expression of Ctrp3, Ctrp9, and Ctrp12 in the visceral fat depot of LFD-fed KO animals. Given the known positive metabolic function of CTRPs in vivo (37-40, 61, 62), these observed changes may, in part, account for the improved glucose metabolism seen in the LFD-fed KO mice. Interestingly, the beneficial effects of improved insulin sensitivity seen in the LFD-fed Tbxas KO mice were largely masked or negated in the KO animals challenged with an HFD; this effect was independent of fasting serum triglycerides, NEFA, adiponectin, and TNF-α levels, and is likely due to other metabolic processes dysregulated by chronic high-fat feeding.

In addition to the adiposity phenotype, chow-fed Cox-2 KO mice also have a striking reduction in the expression of macrophage (Cd68) and inflammatory (Tnfα) markers in adipose tissue (16). In contrast to the COX-2 deficient animals, the expression of markers associated with macrophage number (F4/80 and Cd11c) and polarization (Nos2) was not different in the visceral (epididymal) and subcutaneous (inguinal) fat depot of Tbxas WT and KO mice.

In the study by Saraswathi et al. (48), mice that are transplanted with Cox-1−/− bone marrow cells have reduced immune cell-derived thromboxane B2 (a stable metabolite of thromboxane A2) and elevated fasting blood glucose, triglycerides, and cholesterol levels. Higher blood glucose was partly attributed to reduced
expression and circulating levels of adiponectin, as well as increased gluconeogenic gene expression in the kidney. Intriguingly, gluconeogenic gene expression is decreased in the liver of mice transplanted with Cox-1/- bone marrow cells, opposite to that observed in the kidney of these animals. Insulin signaling is also unexpectedly enhanced in the skeletal muscle of mice receiving Cox-1/- bone marrow cells. Although fasting blood glucose is higher, fasting plasma insulin and HOMA-IR (an index of insulin resistance) are not different between mice transplanted with Cox-1/- or Cox-1/- bone marrow cells (48). Because glucose and insulin tolerance tests were not performed, we do not know if these mice have altered whole-body insulin sensitivity. A second major finding is the observation that mice transplanted with Cox-1/- bone marrow cells have reduced inflammatory marker gene expression without alteration in macrophage number in the adipose tissue, suggesting a possible polarization of adipose tissue macrophages toward the pro-inflammatory M1 phenotype.

In contrast to the Saraswathi et al, study, we found that circulating levels of adiponectin were not different between Tbxas WT and KO mice fed either an LFD or HFD, nor was there any alteration in adipose tissue macrophage number or M1/M2 polarization. It would be interesting, in future studies, to determine whether there are any phenotypic differences between mice transplanted with Cox-1/- or Tbxas/- bone marrow cells. Transplanting TBXAS-deficient bone marrows into lethally irradiated mice will provide additional evidence to further confirm that macrophage-derived thromboxane A2 is indeed responsible for the observed metabolic phenotypes reported in the present study.

It is clear that the absence of thromboxane A2, due to TBXAS deficiency, results in phenotypes that are distinct from mice lacking COX-2 throughout the whole body or mice reconstituted with immune cells devoid of COX-1 enzyme. In both the Ghoshal et al. and Saraswathi et al. studies (16, 48), mice were fed a standard laboratory chow diet and the animals had not been metabolically challenged with an HFD. We do not know if the COX-deficient mice would develop a more pronounced or different metabolic phenotypes when subjected to high-fat feeding. In our study, Tbxas KO mice were fed either an HFD or a control LFD. Both diets were matched for all the micronutrients, and the macronutrients (carbohydrates and fat) were also derived from the same source. The control LFD used in the present study has a lower percent fat content compared to a standard laboratory chow. Thus, the comparisons made between TBXAS- and COX-deficient mice should be tempered by these considerations. In summary, our study provides evidence for the physiological relevance of thromboxane A2 in modulating insulin action and adipose tissue health, functions that are dependent on dietary context.

FOOTNOTES
† Both authors contributed equally.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1. Relative expression of Tbxas and Tbx2r in mice and a macrophage cell line.** A-F, Expression of Tbxas and Tbx2r mRNA expression in: epididymal white adipose tissue (eWAT) of lean wild-type (WT) (n=10) and leptin-deficient obese (ob/ob) (n=10) mice (A-B); eWAT (C-D), subcutaneous (inguinal) white adipose tissue (iWAT) (E-F), liver and skeletal muscle (G, Tbx2r only) of mice fed a low-fat diet (LFD) (n=7) or high-fat diet (HFD) (n=7); and in isolated primary adipocytes or cells of the stromal vascular fraction (SVF) (n=3) (H-I). J-K, Expression of Tbxas and Tbx2r mRNA expression in eWAT and iWAT of chow-fed male mice under fasted, re-fed, or ad libitum conditions (n=10 per group). *P<0.05 compared with ad libitum group. L-M, Expression of Tbxas (L) and Tbx2r (M) mRNA in cultured RAW264.7 macrophage cells treated with recombinant IL-1β (2 ng/mL), IL-6 (2 ng/mL), TNF-α (2 ng/mL), IFN-γ (50 ng/mL), or resistin (100 ng/mL) for 6 h (n=6). Expression data were normalized to 18S rRNA in each sample. All data are expressed as mean ± S.E. *P<0.05; **P<0.01; ***P<0.001.

**Figure 2. Metabolic parameters of Tbxas KO mice fed a low-fat diet (LFD).** A, Body weight gain over time of wild-type (WT) and Tbxas knock-out (KO) male mice fed an LFD (n=10). B, NMR body composition analysis of fat and lean mass in WT and KO mice (n=10). C, Adipose tissue histology (H&E stain) of WT and KO mice. D-I, Expression of Coll (D), Col3 (E) Col6 (F), F4/80 (G), Cd11c (H), and Nos2 (J) in epididymal (eWAT) and inguinal (iWAT) white adipose tissues of Tbxas WT and KO mice (n=10). Expression data were normalized to 18S rRNA in each sample. All data are expressed as mean ± S.E.

**Figure 3. Tbxas deficiency improves glucose homeostasis in low-fed diet (LFD)-fed mice.** A, Food intake measurements of LFD-fed Tbxas WT and KO mice (n=10). B-D, Indirect calorimetry analysis of whole-body oxygen consumption rate (VO2; B), energy expenditure (EE; C), and physical activity (D) of Tbxas WT and KO mice (n=10). E, Blood glucose levels in Tbxas wild-type (WT) and knock-out (KO) mice subjected to an intraperitoneal glucose tolerance test (GTT) (n=8). F, Quantification of cumulative glucose clearance [area-under-curve (AUC)] as shown in E. G, Blood glucose levels in Tbxas WT and KO mice subjected to an insulin tolerance test (ITT) (n=8). H, Quantification of cumulative glucose clearance (AUC) as shown in G. All data are expressed as mean ± S.E. *P<0.05; **P<0.01.

**Figure 4. Serum metabolite and adipokine levels, and metabolic gene expression, in Tbxas KO mice fed a low-fat diet (LFD).** A-E, Fasting serum triglycerides (A), NEFA (B), adiponectin (C), leptin (D), and IL-6 (E) in LFD-fed Tbxas WT and KO mice (n=10). F, Expression of gluconeogenic genes (G6Pc and Pck1) in the liver of WT and KO mice (n=10). G-H, Expression of glucose transporter Glut4 (G) and Ctrp (H) in epididymal (eWAT) and inguinal (iWAT) white adipose tissue of WT and KO mice (n=10). All data are expressed as mean ± S.E. *P<0.05.

**Figure 5. Metabolic parameters of Tbxas KO mice fed a high-fat diet (HFD).** A, Body weight gain over time of wild-type (WT) and Tbxas knock-out (KO) male mice fed an HFD (n=10). B, NMR body composition analysis of fat and lean mass in WT and KO mice (n=10). C, Serum thromboxane B2 (TXB2) in WT and KO mice (n=10). D, Food intake measurements of HFD-fed Tbxas WT and KO mice (n=10). E-G, Indirect calorimetry analysis of whole-body oxygen consumption rate (VO2; E), energy expenditure (EE; F), and physical activity (G) of Tbxas WT and KO mice (n=10). All data are expressed as mean ± S.E. All data are expressed as mean ± S.E. *P<0.05.

**Figure 6. Tbxas deficiency attenuates fibrosis in adipose tissue of high-fat diet (HFD)-fed mice.** A-B, Representative histology images of formalin-fixed, paraffin-embedded tissue sections from epididymal adipose
tissues of HFD-fed *Tbxas* WT and KO mice stained with hematoxylin and eosin (A) or Masson's trichrome (B), which allows visualization of collagen deposition (blue) in extracellular matrices of adipose tissue. Images are shown at 100X and 400X magnification. C-H, Expression of *Col1* (C), *Col3* (D) *Col6* (E), *F4/80* (F), *Cd11c* (G), and *Nos2* (H) in epididymal (eWAT) and inguinal (iWAT) white adipose tissues of *Tbxas* WT and KO mice (n=10). Expression data were normalized to 18S rRNA in each sample. All data are expressed as mean ± S.E. *P<0.05

**Figure 7. Tbxas deficiency has no metabolic impact in high-fat diet (HFD)-fed mice.** A, Blood glucose levels in *Tbxas* wild-type (WT) and knock-out (KO) mice subjected to an intraperitoneal glucose tolerance test (GTT) (n=8). B, Quantification of cumulative glucose clearance [area-under-curve (AUC)] as shown in A. C, Blood glucose levels in *Tbxas* WT and KO mice subjected to an insulin tolerance test (ITT) (n=8). D, Quantification of cumulative glucose clearance (AUC) as shown in C. E-I, Fasting serum triglycerides (E), NEFA (F), adiponectin (G), leptin (H), IL-6 (I), IL-10 (J), MCP-1 (K), and TNF-α (L). All data are expressed as mean ± S.E. *P<0.05
Table 1. Primers for quantitative real-time PCR

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