Thromboxane synthase deficiency improves insulin action and attenuates adipose tissue fibrosis

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Lei X, Li Q, Rodriguez S, Tan SY, Seldin MM, McLenithan JC, Jia W, Wong GW. Thromboxane synthase deficiency improves insulin action and attenuates adipose tissue fibrosis. Am J Physiol Endocrinol Metab 308: E792–E804, 2015. First published March 3, 2015; doi:10.1152/ajpendo.00383.2014.—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 adipose tissue fibrosis. Mice lacking Tbxa2r was detected in adipose stromal cells, including macrophages. Furthermore, stimulation of macrophages with interferon-γ or resistin factors known to be upregulated in obesity induced Tbxa2r expression. Mice lacking Tbxas had similar weight gain, food intake, and energy expenditure. However, loss of Tbxas 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 (Ctpr3, Ctpr9, and Ctpr12) 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 adipose tissue macrophages being affected; however, these changes 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.

adipose tissue; eicosanoid; thromboxane; fibrosis; obesity; diabetes; C1q/tumor necrosis factor-related protein

ARACHIDONIC ACIDS RELEASED from the plasma membrane by phospholipases can be converted to eicosanoids, a class of lipids that includes the prostaglandins, thromboxane, and leukotrienes (28, 50). These signaling lipids play pivotal and pleiotropic 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).

Whereas 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). Whereas 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 mo 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 nonenzymatic pathways, COX-2-derived PGH2 is further converted to other prostanooids with distinct functions, including PGD2, PGE2, PGL2, PGJ2, PGF2α, and thromboxane A2 (60). Thus, in Cox-2-deficient mice, synthesis of multiple types of prostanooids 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 PGH2 to thromboxane A2 (11, 22, 33, 49). Because of 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 Tbxas or Tbx2r in mice results in hemostasis defects (54, 68). Although platelets produce thromboxane in clotting blood, the major source of thromboxane in inflammation is derived from immune cells (23, 57). Mice lacking TBXA2R 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 (Hb A1c). In studies involving obese females with elevated thromboxane levels, weight loss or pioglitazone treatment also result in significant reduction of urinary thromboxane levels (4, 9). Although 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 TNFα, 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.

**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<sup>−/−</sup> mice (all on a C57BL/6J genetic background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were allowed to acclimatize to the animal facility for ≥1 wk. Tbxas<sup>−/−</sup> knockouts (KO) mice and Tbxas<sup>+/−</sup> WT littermate controls were generated by crossing Tbxas<sup>−/−</sup> 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 wk old were used. Laboratory mice consumed standard laboratory chow were used to obtain primary adipocytes and stromal vascular cells [stromal vascular fraction (SVF)] from epididymal adipose tissue.

**Isolation of primary adipocytes and stromal vascular cells from adipose tissue.** Twenty-week-old C57BL/6J WT male mice fed a high-fat diet (HFD; 60 kcal% derived from fat, D12450B; Harlan, Arkon, WI) were used. Male and female Tbxas WT and KO mice were confirmed, weighed, snap-frozen in liquid nitrogen, and stored at −80°C. Blood samples were collected for serum analysis. The fed, fasted, refed experiments in WT C57BL/6J male mice were performed as described previously (39).

**Culture cell.** Mouse 3T3-L1 fibroblasts were cultured and differentiated into adipocytes, as described previously (63). Mouse RAW264.7 macrophage cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s 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), TNFα (2 ng/ml), IFNγ (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 Tbxar2 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 was 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 the manufacturer’s instructions (Applied Biosystems/Life Technologies). Expression was normalized to 18S rRNA for mouse samples. Primer sequences are listed in Table 1. The primer sequences used to assess Ctrp1, Ctrp3, Ctrp9, and Ctrp12 gene expression in adipose tissue have been described previously (62, 65, 66).

**Cell lysis 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 g for 20 min at 4°C to remove insoluble materials. Protein concentration was determined using the Bradford protein assay (Sigma-Aldrich). Twenty micrograms of protein lysate was separated on 10% Bis-Tris NuPAGE gels (Invitrogen). Western blots and quantifications were carried out as described previously (62). Phospho-Akt (Thr<sup>308</sup>) and Akt antibodies were obtained from Cell Signaling Technology.

**Histology.** Formalin-fixed, paraffin-embedded white adipose tissue sections were stained with hematoxylin and eosin at the Pathology Core facility at the Johns Hopkins University School of Medicine. Images were captured with a Zeiss Axiosplan upright microscope with a Zeiss AxioCam 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 tests.** Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as described previously (39) on Tbxas WT and KO mice fed a HFD or LFD for 10–20 wk. For GTT, mice were fasted overnight before ip injection of 1 g glucose/kg body wt. 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 ip injection with 1 U human insulin/kg body wt. Mice were allowed 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<sup>−/−</sup> KO mice from 4 to 24 wk old were used. Laboratory mice consumed

**Table 1. Primers for quantitative real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
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<tbody>
<tr>
<td>Tbxas</td>
<td>TACCATAAGTCGTGTCCTTCCTG</td>
<td>GGCCTGCTGATGGCTCATT</td>
</tr>
<tr>
<td>Tbxar2</td>
<td>GTGCTGTTCCGTGCTCATATTC</td>
<td>CCAACAGTGGATCAATATCG</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>CAGAATTCAAGGATGAGG</td>
<td>GGCCTAAGCTAACCATC</td>
</tr>
<tr>
<td>FG41</td>
<td>TCCGACTTACCTTGTCAG</td>
<td>GGCCTAAGCTAACCATC</td>
</tr>
<tr>
<td>Cdc1l</td>
<td>CTTGCCCTCGGAGCCAT</td>
<td>GGCCTAAGCTAACCATC</td>
</tr>
<tr>
<td>Nos2</td>
<td>TTCCCGAGCGCTAATAAAG</td>
<td>GGCCTAAGCTAACCATC</td>
</tr>
<tr>
<td>Col1</td>
<td>GTCGTCTGTAAAGTGGTGT</td>
<td>AAGCAGCTCATCGGCTG</td>
</tr>
<tr>
<td>Col3</td>
<td>CTTCCGCTGCATTAGAAG</td>
<td>CTTCCGCTGCATTAGAAG</td>
</tr>
<tr>
<td>Col5</td>
<td>CTTGCTGAGAGGAGAAG</td>
<td>CAGGACTGAGAGGAGAAG</td>
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*Tbxa2r, thromboxane synthase; Tbxar2, thromboxane A2 receptor; Col1, -3, and -6, collagen type 1, 3, and 6, respectively.*
insulin/kg body wt. 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. EchoMRI analyses measured fat mass, lean mass, and water content.

**Indirect calorimetry.** Tbxas WT and KO mice (n = 10/group) were used for simultaneous assessments of daily body weight change, energy intake (corrected for spillage), and whole body metabolic production (V\text{CO}_2), physical activity, and food intakes, and from data analysis. Data were collected for 3 days to confirm acclimation to the calorimetry chambers (stable body weights and food intakes), and from data from day 4 in CLAMS were analyzed. Rates of oxygen consumption (V\text{O}_2; ml/kg lean mass \text{^-1-h^-1}), carbon dioxide production (V\text{CO}_2), physical activity, and food intake were measured as described previously (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 AM) from mice that were fasted for 15 h during the dark cycle (7 PM-10 AM). 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, TNFα (Millipore, Billerica, MA), and monocyte chemotactic protein-1 (MCP-1; R & D Systems, Minneapolis, MN) levels were measured by ELISA according to the manufacturer’s instructions. Serum triglycerides and nonesterified free fatty acids were measured using a 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.** Two-way ANOVA and Student’s t-tests were used to determine significant differences between groups. Statistical analyses were performed with GraphPad Prism software (GraphPad Prism, San Diego, CA), and values were considered significant at P < 0.05. All data are presented as means ± SE.

**RESULTS**

**Metabolic perturbations alter Tbxas and Tbxase2r expression in mice.** To establish the physiological relevance of thromboxane A2 to metabolism, we first examined whether changes in metabolic states affect the expression of Tbxas and Tbxase2r. In a genetic model of severe obesity, i.e., leptin-deficient ob/ob mice, Tbxas and Tbxase2r expression was upregulated in epididymal white adipose tissue (Fig. 1, A and B). In a diet-induced obese (DIO) 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); furthermore, epoxyeicosatrienoic acids can act as endogenous antagonists of TBXA2r (5). Based on this consideration, we chose to use Tbxas, rather than Tbxase2r, 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 wk. 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 histologies were 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, and Col6), adipose macrophage (F4/80 and Ccl11), and proinflammatory macrophage M1 (Nos2) marker gene expression in both the visceral and subcutaneous fat depots of Tbxas WT and KO mice (Fig. 2, D–I) and did not observe significant differences between the two groups.

**Tbxas deficiency improves peripheral tissue insulin action in mice fed a LFD.** We next performed indirect calorimetry analysis on LFD-fed mice. No differences were observed in food intake, V\text{O}_2, energy expenditure, or physical activity levels between WT and KO mice (Fig. 3, A–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. 3, E and 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. 3, G and H). Next, we injected insulin into a separate cohort of LFD-fed WT and KO animals to directly evaluating the role of thromboxane A2 in insulin sensitivity.
Fig. 1. Relative expression of thromboxane synthase (\textit{Tbxs}) and thromboxane A2 receptor (\textit{Tbxa2r}) in mice and a macrophage cell line. A–F: expression of \textit{Tbxs} and \textit{Tbxa2r} mRNA expression in epididymal white adipose tissue (eWAT) of lean wild-type (WT; \(n = 10\)) and leptin-deficient obese (\textit{ob/ob}; \(n = 10\)) mice (A and B); eWAT (C and D), subcutaneous (inguinal) white adipose tissue (iWAT; \(E\) and \(F\)), liver, and skeletal muscle (\textit{Tbxa2r} only; \(G\)) of mice fed a low-fat diet (LFD; \(n = 7\)) or high-fat diet (HFD; \(n = 7\)); and isolated primary adipocytes or cells of the stromal vascular fraction (SVF; \(n = 3\)) (\(H\) and \(I\)). \(J\) and \(K\): expression of \textit{Tbxs} and \textit{Tbxa2r} mRNA expression in eWAT and iWAT of chow-fed male mice under fasted, refed, or ad libitum conditions (\(n = 10\)/group). \(L\) and \(M\): expression of \textit{Tbxs} (\(L\)) and \textit{Tbxa2r} (\(M\)) mRNA in cultured RAW264.7 macrophage cells treated with recombinant IL-1\(\beta\) (2 ng/ml), IL-6 (2 ng/ml), TNF\(\alpha\) (2 ng/ml), IFN\(\gamma\) (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 means ± SE. *\(P < 0.05\) compared with ad libitum group; **\(P < 0.01\); ***\(P < 0.001\).
assess the activation of insulin signaling in three major metabolic tissues. Before insulin administration, we observed an approximately twofold 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. Although 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 GTTs and ITTs, appeared to be independent of serum
triglyceride, adiponectin, leptin, and IL-6 levels (Fig. 4, A–E). Only serum nonesterified fatty acid (NEFA) levels were modestly higher in KO mice compared with 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 shown previously 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). Therefore, we also examined the expression of Ctrp in adipose tissue of Tbxas KO mice (Fig. 4H). We have shown previously 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). Therefore, we also examined the expression of Ctrp in adipose tissue of Tbxas KO mice; we focused our analysis on Ctrp1, Ctrp3, Ctrp9, and Ctrp12 based on what was described previously 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 a HFD for a period of 16 wk, we observed no differences in body weight or fat and lean mass between WT and KO mice (Fig. 5, A and B). As expected from Tbxas-deficient mice, serum thromboxane B2, a stable metabolite of thromboxane A2, was largely if not completely abolished compared with WT controls (Fig. 5C). The lowest assay detection limit for thromboxane B2 in mouse serum is 2.4 pg/ml; therefore, we could not distinguish the apparent residual thromboxane B2 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 a HFD also had similar food intake, metabolic rate ($\dot{V}_O_2$), energy expenditure, and physical activity levels compared with WT controls (Fig. 5, D–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). Therefore, we examined the histology and expression of multiple ECM markers in both the visceral (eWAT) and subcutaneous (inguinal WAT (iWAT)) fat depots of Tbxas WT and KO mice.

Histological analysis of adipose tissue sections revealed differences between Tbxas WT and KO mice (Fig. 6, A and B). KO animals had better preservation of adipose tissue architecture, with reduced numbers of stromal vascular cells interspersed among adipocytes (Fig. 6A); the majority of the nonadipocytic cells are infiltrated immune cells, especially macrophages, as has been shown previously (64, 67). Adipose histology also demonstrated decreased deposition of fibrotic collagens (indicated by Masson’s trichrome stain) compared 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. 6, C and D), whereas the adipose expression of Col6 (Fig. 6E) and Hif-1α (not shown) did not differ between the two groups of mice.

Diet-induced obesity is known to result in macrophage infiltration into the adipose compartment (64, 67). Therefore, we 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. 6, F and 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 Tnfa (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 eWAT 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-wk period (data not shown). In contrast to KO mice fed an LFD, we observed no differences in GTT and ITT between Tbxas WT and KO mice fed a HFD (Fig. 7, A–D), nor were there any differences in serum triglycerides, NEFA, adiponectin, IL-10, MCP-1, or TNFα (Fig. 7, E–G and J–L). Interestingly, serum leptin levels were higher and IL-6 levels lower in the KO group relative to WT controls (Fig. 7, H and 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 Tbxas 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). Furthermore, 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. Although 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 be responsible in part 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, whereas 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, and resistin) that help maintain energy homeostasis (45). When adipocyte function is compromised by chronic local

Fig. 6. Tbxas deficiency attenuates fibrosis in adipose tissue of HFD-fed mice. A and 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 ×100 and ×400 magnification. C–H: expression of Col1 (C), Col3 (D), Col6 (E), F4/80 (F), Cd11c (G), and Nos2 (H) in eWAT and 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 means ± SE. *P < 0.05.
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 a LFD or HFD, we observed no differences in the inflammatory state of adipose tissue compared with 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

Fig. 7. Tbxas deficiency has no metabolic impact in HFD-fed mice. A: blood glucose levels in Tbxas WT and KO mice subjected to an intraperitoneal GTT (n = 8). B: quantification of cumulative glucose clearance (AUC), as shown in A. C: blood glucose levels in Tbxas WT and KO mice subjected to an 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), monocyte chemotactic protein (MCP-1; K), and TNFα (L). All data are expressed as means ± SE. *P < 0.05.
deposition in nonadipose 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 (Col1 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 with 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 that 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 PPARγ that plays a critical transcriptional role in orchestrating the adipogenic program (14). The levels of other prostaglandins, including PGD₂, PGE₂, PGI₂, and 6-keto-PGF₁α, are not different in the adipose tissue explants of Cox-2 WT and KO mice (16). We do not know whether 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 mo); 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 a LFD or HFD did not differ in food intake, body weight, or adiposity, although the study duration described here was less than 4 mo.

Despite the striking differences in body weight and adiposity, there appear to be no differences in nonfasting 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 whether the Cox-2 KO mice have improved glucose homeostasis. In contrast to the Cox-2 KO mice, loss of TBXAS improved insulin sensitivity significantly in mice fed a LFD compared with WT controls, as judged by both glucose and insulin tolerance tests. Since circulating levels of leptin, adiponectin, and 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. However, we did observe a higher expression of insulin-responsive Glut4 expression in the adipose tissue of KO mice. Furthermore, 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 account in part 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 a HFD; this effect was independent of fasting serum triglycerides, NEFA, adiponectin, and TNFα 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) or subcutaneous (inguinal) fat depots of Tbxas WT and KO mice.

In a study by Saraswathi et al. (48), mice that were transplanted with Cox-1⁻/⁻ bone marrow cells had 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 attributed partly to reduced expression and circulating levels of adiponectin as well as increased gluconeogeneic gene expression in the kidney. Intriguingly, gluconeogeneic gene expression was decreased in the liver of mice transplanted with Cox-1⁻/⁻ bone marrow cells, which was opposite to that observed in the kidney of these animals. Insulin signaling was 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 whether 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 proinflammatory M1 phenotype. In contrast to the Saraswathi et al. (48) study, we found that circulating levels of adiponectin were not different between Tbxas WT and KO mice fed either a 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 studies by both Ghoshal et al. (16) and Saraswathi et al. (48), mice were fed a standard laboratory chow diet, and the animals had not been metabolically challenged with a HFD. We do not know whether 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 of the micronutrients, and the macronutrients (carbohydrates and fat) were also derived from the same source. The control LFD used in the present study had a lower percent fat.
content compared with 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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