

TRAF3IP2 (TRAF3 Interacting Protein 2) Mediates Obesity-Associated Vascular Insulin Resistance and Dysfunction in Male Mice

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Abstract—Insulin resistance in the vasculature is a characteristic feature of obesity and contributes to the pathogenesis of vascular dysfunction and disease. However, the molecular mechanisms underlying obesity-associated vascular insulin resistance and dysfunction remain poorly understood. We hypothesized that TRAF3IP2 (TRAF3 interacting protein 2), a proinflammatory adaptor molecule known to activate pathological stress pathways and implicated in cardiovascular diseases, plays a causal role in obesity-associated vascular insulin resistance and dysfunction. We tested this hypothesis by employing genetic-manipulation in endothelial cells in vitro, in isolated arteries ex vivo, and diet-induced obesity in a mouse model of *TRAF3IP2* ablation in vivo. We show that ectopic expression of TRAF3IP2 blunts insulin signaling in endothelial cells and diminishes endothelium-dependent vasorelaxation in isolated aortic rings. Further, 16 weeks of high fat/high sucrose feeding impaired glucose tolerance, aortic insulin-induced vasorelaxation, and hindlimb postocclusive reactive hyperemia, while increasing blood pressure and arterial stiffness in wild-type male mice. Notably, *TRAF3IP2* ablation protected mice from such high fat/high sucrose feeding-induced metabolic and vascular defects. Interestingly, wild-type female mice expressed markedly reduced levels of TRAF3IP2 mRNA independent of diet and were protected against high fat/high sucrose diet-induced vascular dysfunction. These data indicate that TRAF3IP2 plays a causal role in vascular insulin resistance and dysfunction. Specifically, the present findings highlight a sexual dimorphic role of TRAF3IP2 in vascular control and identify it as a promising therapeutic target in vasculometabolic derangements associated with obesity, particularly in males.

Graphic Abstract—A graphic abstract is available for this article. (*Hypertension*. 2020;76:1319-1329. DOI: 10.1161/HYPERTENSIONAHA.120.15262.) • **Data Supplement**

Key Words: blood pressure ■ endothelial cell ■ hyperemia ■ insulin resistance ■ sex

Obesity is a leading independent risk factor for cardiovascular diseases (CVDs) and mortality.¹⁻³ It is also strongly associated with insulin resistance and endothelial dysfunction.^{4,5} Insulin, in addition to its role in cellular glucose uptake, increases nitric oxide production from endothelial cells through the PI3K (phosphatidylinositol 3 kinase)/Akt/eNOS (endothelial nitric oxide synthase) pathway.⁶ Notably, mounting evidence supports the notion that vascular insulin resistance manifests early during obesity⁷⁻¹¹ and contributes causally to the development and progression of vascular dysfunction and disease.¹²⁻¹⁷ In this regard, data in humans indicate that impaired insulin signaling in endothelial cells is associated with reduced flow-mediated dilation^{18,19} and increased arterial stiffness.²⁰ Similarly, loss of insulin

signaling in endothelial cells impairs endothelium-dependent relaxation and promotes atherosclerosis in *ApoE*-null mice.²¹ Conversely, restoring endothelial insulin signaling alone prevents impaired endothelium-dependent vasorelaxation in a genetic mouse model of insulin resistance²²; while selective activation of the insulin receptor-PI3K-Akt signaling pathway protects against atherosclerosis development.²³ Despite the indisputable recognition that vascular insulin resistance contributes to the pathogenesis of CVD, the molecular mechanisms underlying vascular insulin resistance in obesity remain largely unknown. A better understanding of such molecular events will help us identify newer therapeutic targets for the treatment of obesity- and insulin resistance-associated vascular diseases.

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TRAF3IP2 (TRAF3 interacting protein 2) is a proinflammatory cytoplasmic adaptor protein that has been identified as a candidate molecule implicated in endothelial dysfunction,²⁴ atherosclerosis,²⁵ and pathological stress pathways in vascular cells.^{26–28} Therefore, we hypothesized that TRAF3IP2 is a key molecular determinant in the development of vascular insulin resistance and dysfunction in obesity. Specifically, using loss-of-function and gain-of-function complementary *in vitro* and *in vivo* experimental approaches, we tested the hypotheses that (1) adenoviral overexpression of TRAF3IP2 in cultured endothelial cells and isolated arteries leads to impaired insulin signaling and depressed endothelium-dependent vasorelaxation, respectively and (2) *TRAF3IP2* deletion protects mice against obesity-associated vascular insulin resistance and dysfunction.

Methods

Extended methods can be found in the [Data Supplement](#). The following references^{29–44} are found in the [Data Supplement](#) only.

Ethics and Approvals

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia. The University of Missouri is accredited by AAALAC International. The data to support the findings of this study can be made available by the corresponding authors on reasonable request.

Experimental Protocols

Experimental Protocol 1: Adenoviral Overexpression of TRAF3IP2 in Cultured Human Endothelial Cells and Isolated Aortic Rings From Wild-Type Mice

Human skeletal muscle microvascular endothelial cells (#H-6220; Cell Biologics; Chicago, IL), human aortic endothelial cells (#CC-2535; Lonza; Allendale, NJ), and isolated aortic rings from 3- to 4-month-old wild-type (WT) male mice (n=11, C57BL/6NTac background; #B6-M, TACONIC, Rensselaer, NY) were used in this protocol. Endothelial cells and isolated aortic rings were cultured in Vasculife EnGS culture medium under standard cell culture conditions (ie, 37°C and 5% CO₂) as previously described.^{31,45} Endothelial cells and aortic rings were transfected with custom generated (Vector Biolabs; Malvern, PA) adenovirus expressing full length human TRAF3IP2 (Ad-GFP-h-TRAF3IP2-MycDDK, 5.7×10⁸ PFU) or mouse TRAF3IP2 (Ad-m-TRAF3IP2, 1.6×10⁸ PFU). Adenovirus expressing GFP (green fluorescent protein, Ad-CMV-GFP; 1.1×10⁸ PFU) served as a control. Cells at 80% to 90% confluency were transfected with respective adenoviruses in Vasculife medium containing 0.5% FBS for ≈18 hours. Following adenoviral incubation, the medium was replaced with fresh medium lacking adenovirus and cultured for an additional 30 hours. Endothelial cells were then treated with or without insulin (100 nM for 30 minutes), washed with cold sterile PBS, and lysed in RIPA buffer (R0278, Millipore Sigma, St. Louis, MO) containing appropriate protease and phosphatase inhibitors. Cell homogenates were then flash frozen for subsequent Western blotting. Skeletal muscle microvascular endothelial cells were selected as our model of choice for insulin signaling experiments because of their high responsiveness to insulin. Aortic rings were incubated in 400 μ L basal Vasculife media (1% FBS, no additional growth factors added) with respective adenovirus for ≈18 hours. The culture medium was then replaced with 500 μ L basal Vasculife medium (1% FBS) without adenovirus for the remaining 30-hour incubation. The aortic rings were then separated from the media and used immediately to measure vasomotor function or flash frozen.

Experimental Protocol 2: TRAF3IP2 Knockout and WT Mice Fed a High Fat/High Sucrose Diet

Both male and female homozygous *TRAF3IP2*-knockout (KO) mice were bred in-house as previously described.⁴⁶ Homozygous

TRAF3IP2-KO mice were originally generated by Dr Siebenlist at TACONIC (Rensselaer, NY) on a C57BL/6NTac background. Age- and sex-matched WT (C57BL/6NTac; #B6-M) mice were purchased from TACONIC. Thus, it should be acknowledged that WT mice were not littermates generated by crossing heterozygous KO mice. At 5 weeks of age, mice were randomized to be fed a chow diet (Picolab 5053) or a high fat/high sucrose (HFHS diet; #F1850, Bio-Serv, Flemington, NJ) for the subsequent 16 weeks. This created 4 experimental groups per sex (n=8–12 mice/group). Samples of the chow and HFHS diets were analyzed for caloric density and macronutrient composition by an independent laboratory (Association of Animal Feed Control Officials Guaranteed Analysis [AAFCO]; EMSL Analytical, Inc, Cinnaminson, NJ); chow diet: 3.00 kcal/g, 59.9% carbohydrate and 14.8% fat kcal/g; HFHS diet: 5.06 kcal/g, 25.0% carbohydrate and 60.1% fat kcal/g. Body weights were measured weekly. Food intake was determined by weighing food in and out over a 3-day period at 4 weeks of the study intervention. Glucose tolerance testing was conducted 14 weeks after diet assignment. Blood pressure and hindlimb postocclusive reactive hyperemia (superficial femoral artery) were assessed 2 weeks before euthanasia. At sacrifice (21 weeks of age), the aorta was excised and used for *ex vivo* assessment of vasomotor function via wire myography, aortic stiffness via atomic force microscopy, or protein expression via Western blotting. The excised femoral artery was used for mechanical testing. Terminal blood collection was performed by cardiac puncture.

Statistical Analyses

GraphPad Prism (version 8.0, GraphPad Prism Software, La Jolla, CA) was used for statistical analysis. Statistical comparisons were performed by *t*-test, univariate or multivariate ANOVA, as appropriate, followed by the Fisher-LSD test for preplanned pairwise comparisons (eg, effect of diet within genotype and effect of genotype within diet). Values are expressed as mean±SEM. A *P*-value <0.05 was considered significant.

Results

TRAF3IP2 Overexpression Impairs Insulin Signaling in Endothelial Cells and Blunts Endothelium-Dependent Relaxation in Isolated Arteries

As displayed in Figure 1A, insulin treatment induced Akt activation (ie, Akt phosphorylation at Ser473/total Akt) in both control and TRAF3IP2-overexpressing cells; however, the level of Akt activation was significantly less in TRAF3IP2-overexpressing cells (*P*<0.05). Total Akt expression was not different between the treatments. Further, as shown in Figure 1B and 1C, adenoviral overexpression of TRAF3IP2 in isolated aortic rings blunted ACh-induced relaxation relative to control GFP expressing rings (*P*<0.05). In comparison, SNP-induced relaxation was not different between the treatment conditions (*P*>0.05). The suppression in ACh-induced aortic vasorelaxation caused by TRAF3IP2 overexpression was in alignment with the downregulation of eNOS in aortic endothelial cells (*P*<0.05), as displayed in Figure 1D.

TRAF3IP2 Deletion Protects Against Obesity-Associated Glucose Intolerance in Male Mice

A representative PCR image of successful *TRAF3IP2* deletion in KO mice is depicted in Figure 2B. Lack of TRAF3IP2 mRNA expression was also confirmed in the heart (Figure 2B). Notably, WT male mice expressed ≈35-fold more TRAF3IP2 mRNA than WT females (*P*<0.05), independent of diet. Sixteen weeks of HFHS feeding led to significant increases in body weight in both male and female mice relative to

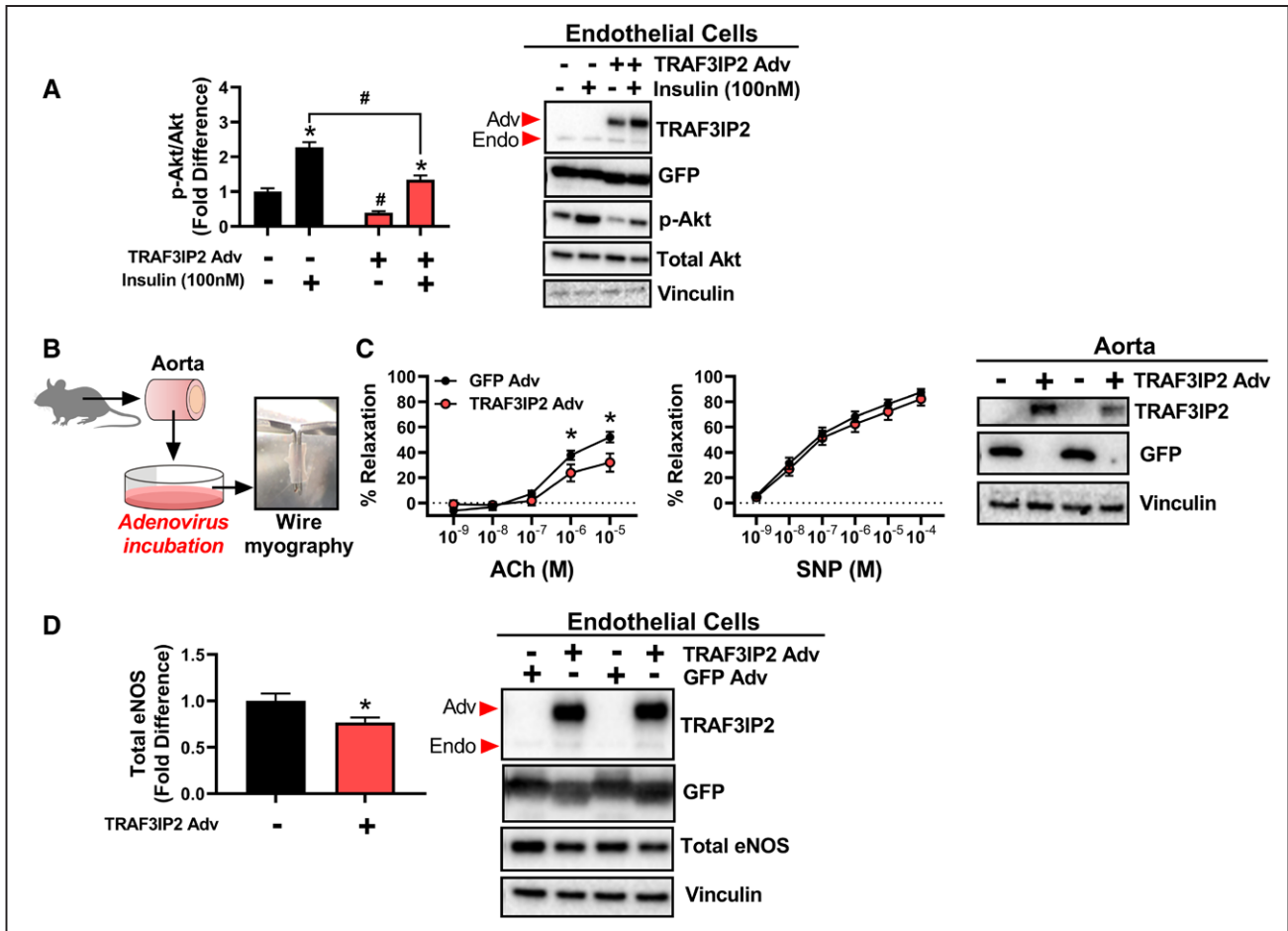


Figure 1. TRAF3IP2 (TRAF3 interacting protein 2) overexpression impairs insulin signaling in endothelial cells and blunts endothelium-dependent relaxation in isolated arteries. Human skeletal muscle microvascular endothelial cells and naïve isolated aortas from wild-type male mice were cultured and transduced with adenovirus (Adv) to overexpress either GFP (green fluorescent protein) or TRAF3IP2 and then treated with or without 100 nM insulin for 30 min. **A**, Ratio of phosphorylated Akt (Ser473, active site) to total Akt levels in cultured endothelial cells under basal conditions and following insulin stimulation; $n=5-6$ /treatment. **B**, Diagram of isolated aorta treatment and utilization. **C**, Endothelium-dependent (acetylcholine, ACh) and -independent (sodium nitroprusside [SNP]) vasorelaxation responses of isolated aortic rings following adenoviral transduction; $n=11$ aortic rings/group. **D**, Basal levels of total eNOS (endothelial nitric oxide synthase) content in cultured human aortic endothelial cells following overexpression of GFP or TRAF3IP2; $n=5-6$ /treatment. Representative Western blot images of successful TRAF3IP2 and GFP overexpression are displayed; Adv arrow indicates adenoviral TRAF3IP2 overexpression band, Endo arrow indicates endogenous TRAF3IP2 band. All values are expressed as mean \pm SEM. * $P<0.05$ vs insulin or adenoviral treatment, # $P<0.05$ vs GFP Adv.

chow-fed counterparts ($P<0.05$, Figure 2C and 2E). Male and female KO mice exhibited greater weight gain from baseline (% change) relative to WT mice, independent of diet (main effect of genotype, $P<0.05$). Final body weights are shown in Table S1 in the [Data Supplement](#). HFHS-fed male and female WT mice consumed 23.4% and 19.9% more calories per day than chow-fed counterparts (main effect of diet, $P<0.05$). HFHS-fed male and female KO mice consumed 21.3% and 18.8% more calories than chow-fed KO mice (main effect of diet, $P<0.05$). Energy intake was not significantly affected by genotype in either males or females, irrespective of the diet ($P>0.05$). However, HFHS feeding increased fasting insulin concentrations ($P<0.05$, Figure 2D and 2F) and impaired glucose tolerance in male and female WT mice compared with chow-fed mice ($P<0.05$, Figure 2D and 2F). Moreover, TRAF3IP2 deletion (KO) ameliorated HFHS-induced glucose intolerance only in males ($P<0.05$, Figure 2D). Fasting plasma glucose concentrations were increased with HFHS feeding in both males and females, whereas HbA1c was only increased in HFHS-fed males ($P<0.05$, Table S1).

TRAF3IP2 Deletion Protects Against Obesity-Associated Impairments in Insulin-Induced Vasorelaxation in Male Mice

As shown in Figure 3A, HFHS feeding significantly blunted insulin-induced aortic vasorelaxation in male WT ($P<0.05$), but not KO, mice ($P>0.05$). ACh-induced aortic vasorelaxation was not significantly affected by diet ($P>0.05$); however, it was greater in HFHS-fed male KO mice relative to WT counterparts ($P<0.05$). HFHS feeding modestly reduced SNP-induced aortic vasorelaxation in male WT ($P<0.05$), but not KO, mice ($P>0.05$). SNP-induced relaxation was increased in HFHS-fed male KO mice relative to HFHS-fed WT mice ($P<0.05$). Furthermore, male HFHS-fed WT mice exhibited hyperresponsiveness to phenylephrine, an α_1 -adrenergic receptor vasoconstrictor, compared with chow-fed counterparts ($P<0.05$, Figure 3B). Notably, this dietary effect was absent in KO mice ($P>0.05$). Aortic Akt activation (ie, Ser473 phosphorylation) and eNOS content were not affected by HFHS feeding in male WT mice ($P>0.05$). However, male KO mice fed HFHS exhibited a heightened Akt activation, which coincided

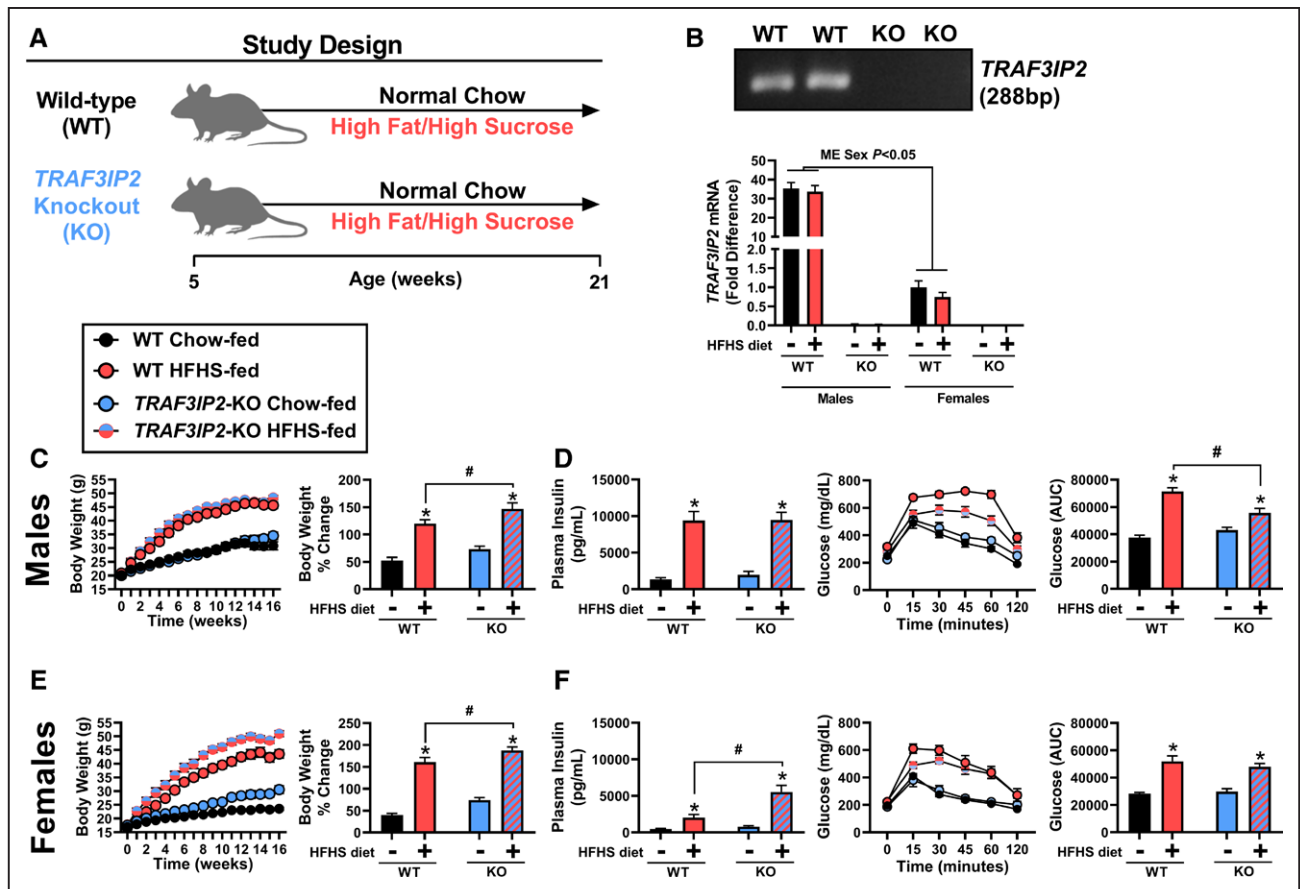


Figure 2. *TRAF3IP2* deletion protects male mice against obesity-associated glucose intolerance. **A**, Graphical depiction of the study design. Male and female wild-type (WT) or *TRAF3IP2*-knockout (KO) mice were fed either a normal chow or high fat/high sucrose (HFHS) diet for 16 wk. **B**, Representative image of PCR gel depicting ablation of *TRAF3IP2* gene (indicated at 288 base pairs [bp]) in KO mice and qPCR determination of *TRAF3IP2* mRNA transcripts in heart homogenates from male and female WT or KO mice; $n=8-11$ /group. **C**, Changes in body weight over time and as percent change from baseline in male mice; $n=8-10$ /group. **D**, Fasting plasma insulin in male mice; $n=8-10$ /group; glycemic excursions over time during a glucose tolerance test (GTT) and glucose area under the curve (AUC) in male mice; $n=8-10$ /group. **E**, Changes in body weight over time and as percent change from baseline in female mice; $n=10-12$ /group. **F**, Fasting plasma insulin in female mice; $n=10-12$ /group; Glycemic excursions over time during a GTT and glucose AUC in female mice; $n=10-12$ /group. All values are expressed as mean \pm SEM. * $P<0.05$ vs chow-fed counterpart (within genotype); # $P<0.05$ vs WT counterpart (within diet); main effect (ME) of sex included in (C).

with the induction of total eNOS content, relative to all other groups ($P<0.05$, Figure 3C).

Vasomotor reactivity data in females are summarized in Figure S1A and S1B. Contrary to findings in males, both WT and KO female mice were protected against HFHS feeding-associated impairments in aortic vasorelaxation responses to insulin. Similarly, ACh- and SNP-induced aortic vasorelaxation were not influenced by diet or genotype in female mice. Phenylephrine-induced aortic vasoconstriction was also not affected by diet in female WT or KO mice. However, female chow-fed KO mice exhibited hyporeactivity to phenylephrine relative to chow-fed WT mice ($P<0.05$).

***TRAF3IP2* Deletion Protects Against Obesity-Associated High Blood Pressure, Arterial Stiffening, and Impairments in Hindlimb Postocclusive Reactive Hyperemia in Male Mice**

As depicted in Figure 4A, HFHS feeding increased blood pressure in male WT ($P<0.05$), but not KO, mice ($P>0.05$). Male KO mice were also protected against HFHS feeding-induced aortic endothelial cortical stiffening as assessed by

atomic force microscopy ($P<0.05$, Figure 4B) and exhibited reduced femoral artery stiffness as determined by a rightward shift in the strain-stress curve ($P<0.05$, Figure 4C). In male WT, but not male KO mice, HFHS feeding suppressed hindlimb postocclusive reactive hyperemia ($P<0.05$, Figure 4D through 4F). However, female HFHS-fed KO mice did exhibit increased blood pressure relative to chow-fed counterparts ($P<0.05$, Figure S1C).

Proteomic Analysis of Aortas From Male Mice

Proteomic analysis of aortas from male WT and KO mice detected 1890 proteins. Comparisons between the WT chow-fed and WT HFHS-fed groups identified 119 proteins that were differentially expressed (Figure 5A; Table S2). Additional comparisons between HFHS-fed WT and KO groups identified 143 proteins that were differentially expressed (Figure 5A; Table S3). Of note, Akt1 was increased (1.7-fold, $P=0.038$) in aortas from HFHS-fed KO mice relative to HFHS-fed WT mice. Using K-means Clustering within the HFHS-fed KO versus WT comparison, 4 protein network clusters were identified and consisted of 28 total proteins

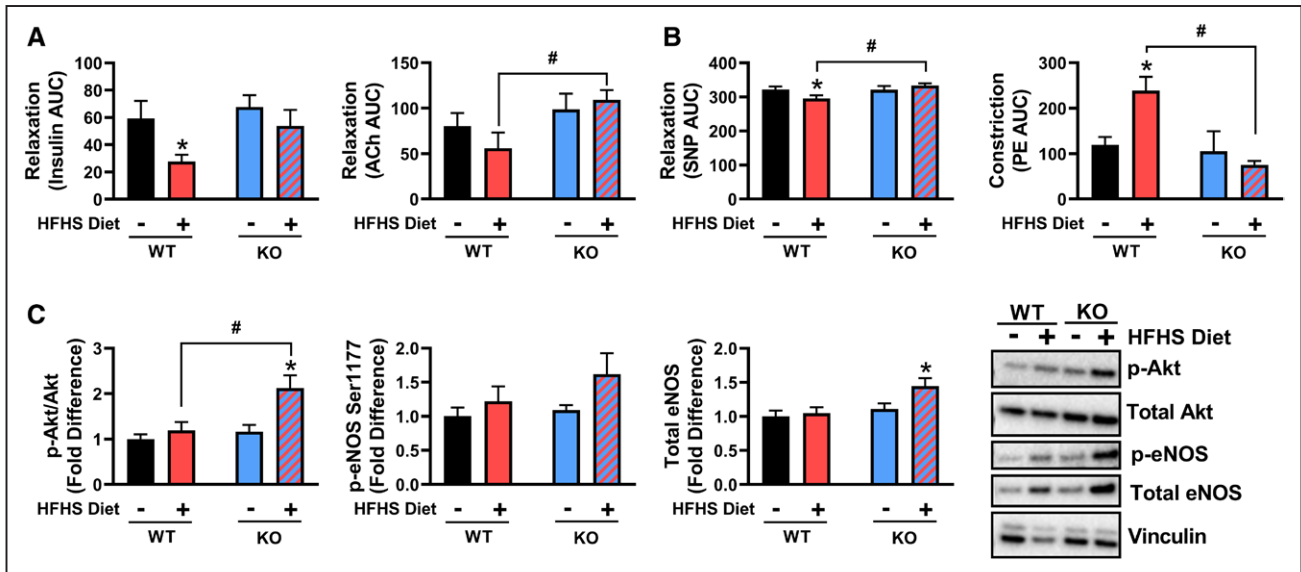


Figure 3. *TRAF3IP2* deletion protects male mice against obesity-associated impairments in insulin-induced vasorelaxation. **A**, Aortic vasorelaxation responses to insulin, acetylcholine (ACh), and sodium nitroprusside (SNP) following preconstriction with U-46619 (20 nM) in male wild-type (WT) and *TRAF3IP2* knockout (KO) mice fed either a chow or high fat/high sucrose (HFHS) diet for 16 wk; $n=8-10$ /group. **B**, Aortic vasoconstriction responses to phenylephrine (PE). **C**, Determination of Akt activation (p-Akt Ser473/total Akt), eNOS (endothelial nitric oxide synthase) activation (p-Ser1177), and total eNOS in aortic rings from WT and KO mice; representative Western blot images are displayed; $n=7-10$ /group. All values are expressed as mean \pm SEM. * $P<0.05$ vs chow-fed counterpart (within genotype); # $P<0.05$ vs WT counterpart (within diet).

(Figure S2). In addition, 12 select statistically significant proteins with potential cardiovascular relevance were identified from the quantitative proteomic analysis. Accordingly, 40 candidate proteins (listed in Table S4) were input into the STRING resource software and the resulting protein network diagram is displayed in Figure 5B.

Discussion

Insulin resistance in the vasculature is a distinguishing feature of obesity and contributes to the pathogenesis of vascular dysfunction and disease. However, the molecular mechanisms underlying vascular insulin resistance and dysfunction in obesity have remained largely unknown. Here, for the first time, we provide evidence that the proinflammatory adaptor molecule TRAF3IP2 plays a causal role in obesity-associated vascular insulin resistance and dysfunction, specifically in male mice.

We report that TRAF3IP2 overexpression by adenoviral transduction blunts insulin signaling (ie, Akt activation) in cultured endothelial cells and depresses endothelium-dependent vasorelaxation in isolated aortic rings. Further, we found that male *TRAF3IP2*-KO mice are protected against HFHS diet-induced glucose intolerance and vascular insulin resistance, despite greater weight gain, relative to WT counterparts. These findings expand upon our prior work indicating that TRAF3IP2 mediates atherosclerotic plaque development in *ApoE*-null mice²⁵ and the impairment in ACh-induced vasorelaxation caused by acute oxidized LDL exposure.²⁴ Vascular insulin resistance represents an early event in obesity⁷⁻¹¹ and a causal factor in the pathogenesis of CVD.¹²⁻¹⁷ Thus, the identification of TRAF3IP2 as a novel mediator of vascular insulin resistance is of utmost clinical importance and may be key to identifying newer strategies for the prevention and treatment of vascular diseases associated with obesity and insulin resistance.

It is well established that diet-induced obesity is associated with increases in blood pressure^{47,48} and vascular stiffness⁴⁷⁻⁵⁰; yet, the molecular mechanisms underlying these vascular derangements in obesity have yet to be fully discerned. Importantly, arterial stiffening is a significant risk factor for the development of hypertension^{51,52} with previous work indicating that diet-induced arterial stiffening may in fact precede the onset of overt hypertension.⁴⁷ Here, in agreement with our hypothesis, we show that *TRAF3IP2*-KO male mice are protected against HFHS diet-induced increases in blood pressure and arterial stiffness. Furthermore, we report that HFHS diet-induced aortic hyperreactivity to phenylephrine, an α_1 -adrenergic receptor agonist, was prevented by the loss of *TRAF3IP2*. Such attenuated α_1 -adrenergic receptor-mediated vasoconstriction in *TRAF3IP2*-deficient male mice may contribute to their normotensive phenotype despite obesity.⁵³⁻⁵⁵ In addition, given the known relationships between insulin resistance, arterial stiffness, and hypertension, the reduced blood pressure and arterial stiffness in male HFHS-fed KO mice may also be attributed to preserved vascular insulin sensitivity.^{14,56-58} Collectively, our findings for the first time support an important role for TRAF3IP2 in blood pressure control and arterial stiffening in the setting of obesity.

Growing evidence indicates that perturbations in microvascular function are linked to the development of insulin resistance, hypertension, and arterial stiffening in obesity.⁵⁹⁻⁶² One of the most widely utilized assessments of resistance vessel function in humans is postocclusive reactive hyperemia via Doppler ultrasound.⁶³ In fact, impaired reactive hyperemia is considered an independent predictor of adverse cardiovascular events and a well-established surrogate marker of CVD risk.^{36,63-67} Although the assessment of postocclusive reactive hyperemia in small animal models has been challenging, because of their reduced vascular dimensions, advancements in ultrasound technology have made visualization and detection of conduit arteries in rodents

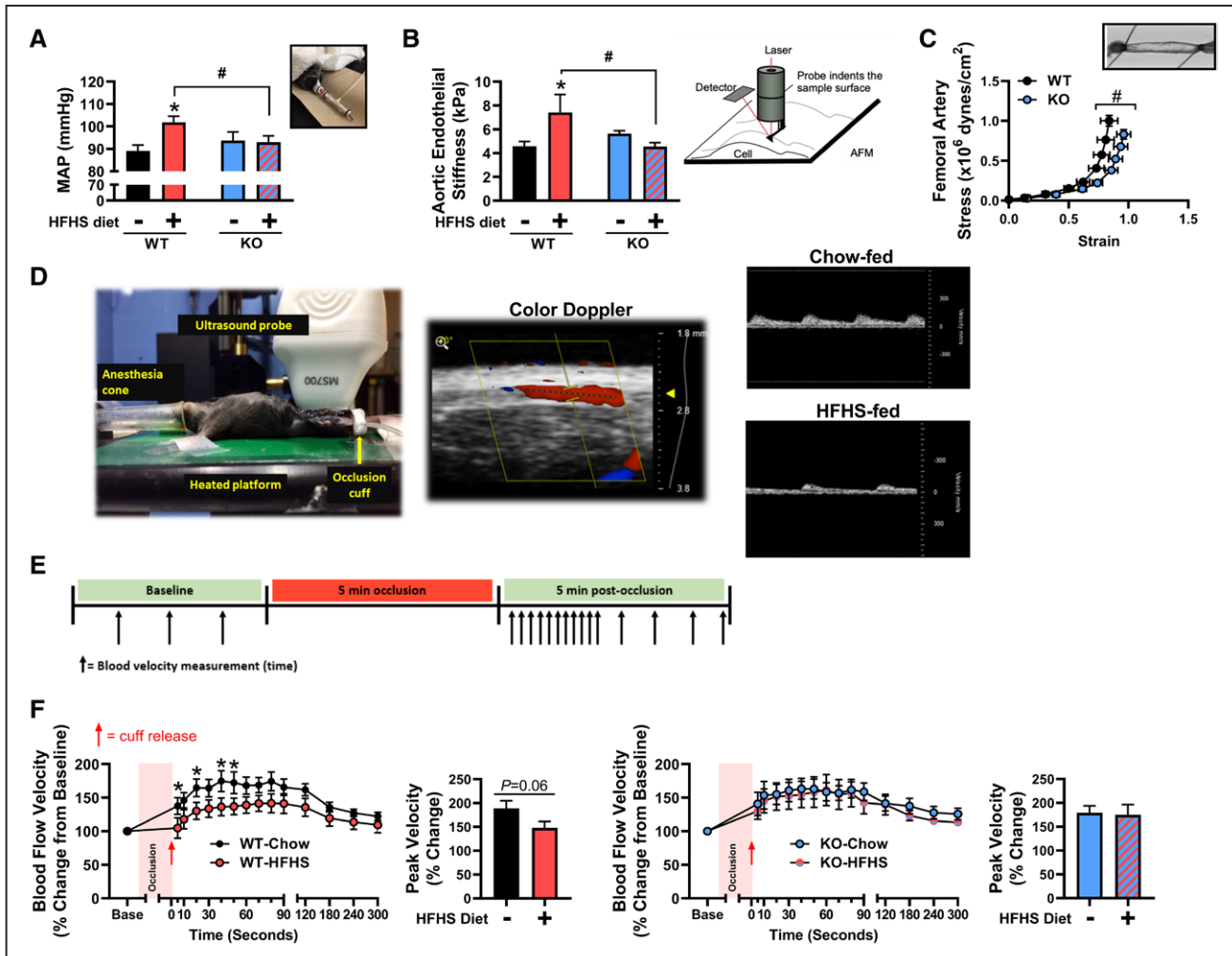


Figure 4. *TRAF3IP2* deletion protects male mice against obesity-associated high blood pressure, arterial stiffening and impairments in hindlimb post-occlusive reactive hyperemia. **A**, Mean arterial blood pressure as determined by tail-cuff plethysmography in wild-type (WT) and *TRAF3IP2* knockout (KO) mice fed either a chow or high fat/high sucrose (HFHS) diet; $n=8-10$ /group. **B**, Aortic endothelial cortical stiffness as assessed by atomic force microscopy (AFM) and graphical representation of instrumental setup; $n=6-8$ /group. **C**, Femoral artery stiffness as determined by strain-stress analysis in arteries pressurized under passive conditions; $n=18-19$ /group (diets collapsed). **D**, Depiction of ultrasonography set up for the assessment of postocclusive reactive hyperemia in the superficial femoral artery in mice. Representative images of peak blood velocity during reactive hyperemia in chow-fed and HFHS-fed mice are displayed. **E**, Graphical representation of the reactive hyperemia protocol and assessment of blood velocity over time. Percent (%) change of femoral artery blood velocity over time during reactive hyperemia in **(F)** WT and KO mice fed either a chow or HFHS diet and peak blood velocity during reactive hyperemia; $n=8-10$ /group. All values are expressed as mean \pm SEM. * $P<0.05$ vs chow-fed counterpart (within genotype); # $P<0.05$ vs WT counterpart (within diet).

possible. Indeed, 2 reports have validated the in vivo assessment of postocclusive reactive hyperemia in mice³⁴ and rats.³⁵ Consistent with our hypothesis, we found that loss of *TRAF3IP2* protects against HFHS diet-induced impairments in hindlimb reactive hyperemia, underscoring a hitherto unidentified novel role for *TRAF3IP2* in microvascular control. We have previously demonstrated that *TRAF3IP2* is a mediator of endothelin-1 production,²⁷ a vasoconstrictor peptide primarily released from endothelial cells and a known regulator of reactive hyperemia.⁶⁸ Since obesity exacerbates endothelin-1 actions,⁶⁹⁻⁷¹ it is plausible that *TRAF3IP2* ablation protects against HFHS diet-induced impairments in hindlimb reactive hyperemia by limiting microvascular endothelin-1 production/function. However, further research is warranted to better understand the relationship between *TRAF3IP2* and endothelin-1 in microvascular control.

An additional important finding of the current investigation is that *TRAF3IP2* regulates Akt activation and expression.

Notably, *TRAF3IP2* is a known regulator of stress-activated protein kinases and inflammatory signaling (ie, IKK/NF- κ B and JNK/AP-1 signaling) in various cell types,⁷²⁻⁷⁴ including vascular tissues.²⁵⁻²⁸ Namely, we have previously shown that overexpression of *TRAF3IP2* increases IKK/NF- κ B and JNK/AP-1 activation in endothelial cells,²⁷ while silencing of *TRAF3IP2* limits high glucose- and IL-17-induced inflammation in endothelial^{26,27} and vascular smooth muscle cells,²⁸ respectively. However, whether *TRAF3IP2* regulates Akt, an important molecular determinant of insulin signaling, endothelium-dependent function, and overall vascular control⁷⁵⁻⁷⁹ has yet to be determined. Here, we provide the first evidence that overexpression of *TRAF3IP2* limits insulin-induced Akt activation in endothelial cells and that genetic ablation of *TRAF3IP2* enhances Akt activation in hyperinsulinemic male mice (ie, mice-fed HFHS). In addition, the aortic proteomic analysis revealed an increase in Akt1 expression, the

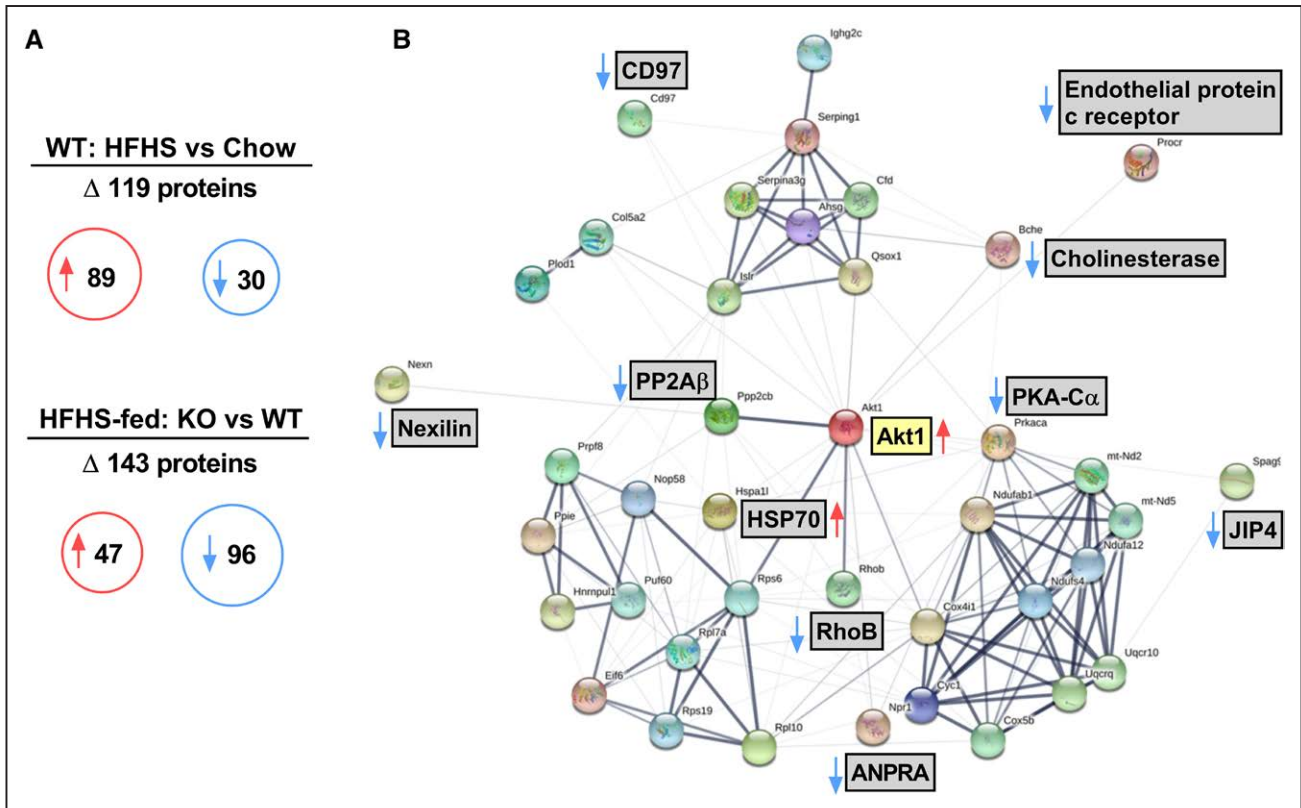


Figure 5. Proteomic analysis and network interactions of proteins identified in aortic tissues from male mice. **A**, Quantitative proteomic analysis of differentially expressed proteins between male chow-fed *TRAF3IP2* knockout (KO) vs wild-type (WT) and high fat/high sucrose (HFHS)-fed KO and WT mice; $n=8-10$ samples analyzed/group. In summary, 1890 proteins were identified with 119 proteins differentially expressed between chow and HFHS-fed WT mice (89 proteins increased, 30 proteins decreased) and 143 proteins differentially expressed between HFHS-fed KO and WT mice (47 proteins increased, 96 proteins decreased). **B**, Generated protein network interactions from 40 target proteins of interest identified in the quantitative proteomic analysis between HFHS-fed KO and HFHS-fed WT mice. Thicker bands indicate increased strength of data to support the observed protein-protein interactions as generated per the STRING resource software. Red arrows indicate increased protein expression and blue arrow indicate decreased protein expression in HFHS-fed KO mice relative to HFHS-fed WT mice. Gene names are located next to each protein node. Full protein/gene details can be found in Table S4 in the [Data Supplement](#). ANPRA indicates atrial natriuretic peptide receptor 1; HSP70, heat shock protein 70; JIP4, C-Jun-amino-terminal kinase-interacting protein 4; PKA-C α , protein kinase A catalytic subunit α ; PP2A β , Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform; and RhoB, Rho-related GTP-binding protein RhoB.

dominant Akt isoform active in cardiovascular tissues,⁸⁰⁻⁸² in male *TRAF3IP2*-KO mice fed HFHS. Therefore, given the known roles of Akt in metabolic and vascular control, it is plausible that increases in vascular Akt activation and expression mediate vascular protection against obesity in *TRAF3IP2*-deficient mice. Stated differently, it seems that the loss of *TRAF3IP2* unrestricts Akt signaling in the setting of hyperinsulinemia and obesity. Because prolonged insulin stimulation and signaling through Akt leads to increased eNOS expression,⁸³⁻⁸⁵ it is not surprising that increased Akt activation was accompanied with an induction of total eNOS content in aortic homogenates of hyperinsulinemic/obese mice lacking *TRAF3IP2*, likely further contributing to their overall preserved vascular function. These findings are highly significant in that selective activation of the PI3K/Akt/eNOS branch of the insulin signaling pathway is associated with improved vascular outcomes in a model of obesity and metabolic syndrome.²³

To further identify additional potential adaptive molecular mechanisms resulting from the loss of *TRAF3IP2* in male HFHS-fed mice, we used the STRING resource software to identify potential protein-protein interaction networks among 40 candidate proteins selected from the proteomic analysis.

Interestingly, the STRING resource software generated an Akt1-centric network diagram (Figure 5B). This observation indicates that many of the differentially expressed proteins resulting from *TRAF3IP2* ablation interact with Akt, thus further supporting the notion that increased Akt signaling may be a mechanism by which loss of *TRAF3IP2* protects against obesity-associated vascular dysfunction. In this regard, HSP70 (heat shock protein 70), Rho-related GTP-binding protein RhoB, PKA-C α (protein kinase A catalytic subunit α), identified in Figure 5B, are known transducers of Akt activation and sequestration in vascular tissue⁸⁶⁻⁸⁸ and are likewise implicated in vascular health and function.^{89,90} Nevertheless, more research is warranted to delineate the molecular mechanisms by which *TRAF3IP2* directly or indirectly regulate Akt activation and signaling.

Another important observation of this current investigation is that female mice exhibited a largely protected vascular phenotype following 16 weeks of HFHS feeding. Notably, this protection coincided with the finding that female mice displayed a ≈ 35 -fold lower *TRAF3IP2* mRNA expression compared with males. Thus, it is likely that the apparent vascular protection in our female WT mice might be because of low levels of *TRAF3IP2* expression. Along similar lines, given the low

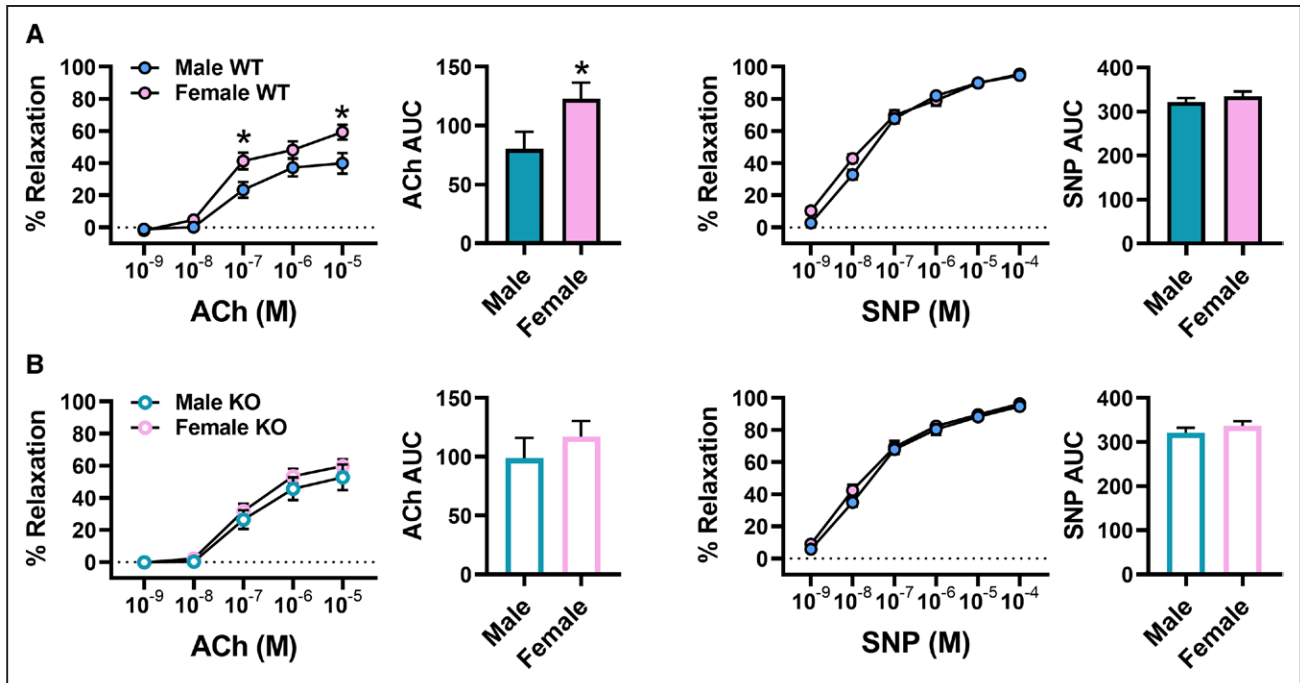


Figure 6. Wild-type (WT) male mice exhibit lower endothelium-dependent vasorelaxation relative to females. Comparison of aortic vasorelaxation responses to acetylcholine (ACh; endothelium-dependent) and sodium nitroprusside (SNP; endothelium-independent) between male and female (A) WT and (B) KO mice fed chow; n=8–12/group. All values are expressed as mean±SEM. * $P<0.05$ vs males.

basal expression of TRAF3IP2 in females, it is not surprising that genetic ablation of *TRAF3IP2* seems to be less consequential to their vascular function. To further explore the impact of sex and TRAF3IP2 on vasomotor function, we conducted a secondary analysis directly comparing endothelium-dependent and -independent relaxation responses between males and females. As shown in Figure 6, WT males (high TRAF3IP2 expressors) exhibit lower endothelium-dependent relaxation relative to females (low TRAF3IP2 expressors), while endothelium-independent relaxation remains similar between sexes. Notably, this sex difference in endothelial function is abolished in mice lacking *TRAF3IP2*, further underscoring the role of TRAF3IP2 in mediating sex differences in vascular function. Future research should explore the relationship between sex and TRAF3IP2 throughout the lifespan as it is known that women exhibit increased susceptibility to CVD with age.⁹¹ Importantly, these findings add to the emergent literature supporting the notion that sex should be deemed as a key variable when identifying the molecular mechanisms underlying CVD.

There are some limitations to our work that should be given consideration. While our HFHS diet produced the intended metabolic and vascular effects, as also recently reported by others,⁴⁸ we acknowledge that the use of the standard grain-based chow diet is not the most appropriate control diet against the employed HFHS, which is a purified ingredient diet. Accordingly, future work would benefit from utilizing a matching low-fat purified ingredient diet as the control to the present HFHS diet administered. Indeed, previous reports have found that matching and controlling for purified ingredients and micronutrients between low-fat and high-fat diets can influence differences in metabolic and health outcomes in rodents and thus the interpretation of the findings.^{92–95} Further, consideration should be given to our glucose administration during the GTT and the use of tail-cuff

plethysmography. In the present study, the glucose load utilized during the GTT may have overestimated the level of glucose intolerance in HFHS-fed mice as these mice were given a greater glucose load based on body weight. Notably, mice lacking *TRAF3IP2* and fed a HFHS diet tended to be heavier than WT counterparts. Interestingly, they still displayed better glucose disposal. Along these lines, the protective metabolic phenotype displayed by this mouse model should be further interrogated by incorporating detailed measures of body composition as well as determining energy expenditure and spontaneous physical activity using metabolic cages. Last, although we found significant blood pressure differences between male HFHS-fed WT and KO mice using a tail-cuff blood pressure method that has been previously validated,⁹⁶ future research should confirm these findings using telemetry-based methodologies.

Perspectives

Insulin resistance in the vasculature is a characteristic feature of obesity and contributes to the pathogenesis of vascular dysfunction and disease. Findings from the present study demonstrate that TRAF3IP2, a proinflammatory adaptor molecule, plays a causal role in obesity-associated vascular insulin resistance and dysfunction. Moreover, our studies highlight a sexual dimorphic role of TRAF3IP2 in vascular control and identify it as a promising therapeutic target in vasculometabolic derangements associated with obesity, particularly in males.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Ectopic expression of TRAF3IP2 (TRAF3 interacting protein 2) blunts insulin signaling (ie, Akt activation) in cultured endothelial cells and depresses endothelium-dependent vasorelaxation in isolated aortic rings.
- TRAF3IP2 deletion protects against obesity-associated impairments in glucose tolerance and insulin-induced vasorelaxation in male mice.
- TRAF3IP2 deletion protects against obesity-associated high blood pressure, arterial stiffening, and impairments in hindlimb postocclusive reactive hyperemia in male mice.
- TRAF3IP2 regulates Akt activation and expression.

What Is Relevant?

- Vascular insulin resistance contributes to the pathogenesis of vascular diseases.
- Here, we demonstrate that TRAF3IP2 mediates obesity-associated vascular insulin resistance and dysfunction in male mice.

Summary

TRAF3IP2 plays a causal role in obesity-associated vascular insulin resistance and dysfunction. Moreover, our findings highlight a sexual dimorphic role of TRAF3IP2 in vascular control and identify it as a promising therapeutic target in vasculometabolic derangements associated with obesity, particularly in males.