Contribution of Insulin and Akt1 Signaling to Endothelial Nitric Oxide Synthase in the Regulation of Endothelial Function and Blood Pressure

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Abstract—Impaired insulin signaling via phosphatidylinositol 3-kinase/Akt to endothelial nitric oxide synthase (eNOS) in the vasculature has been postulated to lead to arterial dysfunction and hypertension in obesity and other insulin resistant states. To investigate this, we compared insulin signaling in the vasculature, endothelial function, and systemic blood pressure in mice fed a high-fat (HF) diet to mice with genetic ablation of insulin receptors in all vascular tissues (TTr-IR−/−) or mice with genetic ablation of Akt1 (Akt1−/−). HF mice developed obesity, impaired glucose tolerance, and elevated free fatty acids that was associated with endothelial dysfunction and hypertension. Basal and insulin-mediated phosphorylation of extracellular signal-regulated kinase 1/2 and Akt in the vasculature was preserved, but basal and insulin-stimulated eNOS phosphorylation was abolished in vessels from HF versus lean mice. In contrast, basal vascular eNOS phosphorylation, endothelial function, and blood pressure were normal despite absent insulin-mediated eNOS phosphorylation in TTr-IR−/− mice and absent insulin-mediated eNOS phosphorylation via Akt1 in Akt1−/− mice. In cultured endothelial cells, 6 hours of incubation with palmitate attenuated basal and insulin-stimulated eNOS phosphorylation and NO production despite normal activation of extracellular signal-regulated kinase 1/2 and Akt. Moreover, incubation of isolated arteries with palmitate impaired endothelium-dependent but not vascular smooth muscle function. Collectively, these results indicate that lower arterial eNOS phosphorylation, hypertension, and vascular dysfunction following HF feeding do not result from defective upstream signaling via Akt, but from free fatty acid–mediated impairment of eNOS phosphorylation. (Circ Res. 2009;104:1085-1094.)

Key Words: arterial insulin signaling ■ hypertension ■ endothelial dysfunction ■ mice ■ diabetes

Stimulation of insulin receptors in the vasculature leads to increased activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the mitogen-activated protein (MAP) kinase (eg, extracellular signaling-regulated kinase [ERK1/2]) pathway. Insulin receptor (IR)-mediated stimulation of PI3K/Akt leads to endothelial nitric oxide (NO) synthase (eNOS) phosphorylation, NO production, and vasorelaxation. Insulin-mediated activation of ERK1/2 leads to endothelin (ET)-1 production, inhibition of eNOS phosphorylation, and subsequent vasocontraction.

Evidence from several experimental models of insulin resistance reveals impaired insulin-mediated PI3K/Akt-dependent signaling in the vasculature, whereas ERK1/2 pathways are preserved or even augmented. Collectively, these observations have led to the hypothesis that an imbalance in vascular insulin-mediated signaling can precipitate cardiovascular complications including endothelial dysfunction and hypertension. Recently, it was shown that insulin-mediated Akt phosphorylation was preserved, but NO-mediated vasorelaxation was blunted, in arteries from obese, glucose intolerant versus lean mice, suggesting that impaired insulin-mediated signaling to eNOS via Akt might not be required to reduce NO bioavailability to an extent that produces endothelial dysfunction. Cardiovascular consequences of decreased insulin-mediated signaling have been evaluated in mice homozygous for IR deletion in the endothelium (VENIRKO mice) and in heterozygous mice with germline IR deletion (VENIRKO mice). Vascular eNOS and ET-1 mRNA were lower in VENIRKO versus wild-type (WT) mice, blood pressure was similar between groups, but eNOS protein and phosphorylation levels were not reported. In contrast, eNOS mRNA was unchanged, blood pressure was elevated, and insulin-mediated eNOS phosphorylation in the vasculature and indi-
ces of NO bioavailability were reduced in heterozygous IRKO versus WT animals. Heterozygous IRKO mice are glucose-intolerant and hyperinsulinemic, which may secondarily influence vascular function. Thus, the specific contribution from impaired vascular insulin-mediated signaling to the pathogenesis of arterial dysfunction and hypertension is unclear.

In light of the uncertainty concerning the cardiovascular consequence(s) of insulin resistance in the vasculature, we compared insulin-mediated signal transduction to eNOS in the vasculature, endothelial function, and blood pressure between mice that consumed high-fat (HF) and standard chow (CON). Our findings indicated that endothelial dysfunction and hypertension associated with diet-induced obesity could not be explained by defective upstream signaling via Akt to eNOS in the vasculature. To validate these observations, we used 2 genetic models of “insulin resistance” that lack systemic metabolic disturbances associated with obesity and type 2 diabetes. First, IR-null mice with transgenic reexpression of the IR in brain, liver, and pancreatic β-cells (TTr-IR+/− mice) were used to test the consequence of absent insulin-mediated signaling to eNOS in the vasculature, endothelial function, and blood pressure. Second, Akt1-null mice (Akt1−/− mice) were used to evaluate the contribution of insulin-mediated signaling via Akt1 to eNOS on vascular function and blood pressure. Akt1−/− mice were chosen because this isoform was recently shown to be most important in regulating eNOS, based on observations that basal and vascular endothelial cell growth factor (VEGF)-stimulated NO production were reduced in endothelial cells isolated from mice with deletion of Akt1 but not Akt2.

Findings from these genetic models suggested that disruption of insulin or Akt1 signaling to eNOS in the vasculature does not precipitate endothelial dysfunction or hypertension. Because the cardiovascular complications observed in mice with diet-induced obesity might not result from impaired vascular insulin-mediated signal transduction, we sought to determine the role of elevated circulating free fatty acids (FFAs). When bovine aortic endothelial cells (BAECs) were incubated with the saturated FFA palmitate, both basal and insulin-mediated eNOS phosphorylation and NO production were impaired despite normal activation of Akt and ERK. Furthermore, palmitate incubation precipitated endothelium-dependent dysfunction in isolated vessels. Collectively, these results indicate that reduced arterial eNOS phosphorylation and vascular complications do not result from defective upstream signaling via Akt but might be secondary to FFA-mediated impairment of eNOS phosphorylation.

Materials and Methods
All protocols were approved by the Institutional Animal Care and Use Committee. Ten-week-old C57Bl6 mice that consumed standard (CON) or HF chow for 10 to 12 weeks were used for experiments on metabolic characterization, insulin-mediated signal transduction in the vasculature, blood pressure, vascular function, detection of vascular oxidant load, and mRNA expression. Similar experiments were completed in TTr-IR+/−, Akt1−/− mice, and their WT littermates. Detailed procedures are provided in the expanded Materials and Methods section in the online data supplement at http://circres.ahajournals.org.

Statistics
Data are presented as means±SEM. Significance was accepted at P<0.05. Comparison of 1 time point between 2 groups was made...
using an unpaired t test. Comparison of multiple time points between groups was made using a 1-way or 2-way repeated measures ANOVA. Tukey post hoc tests were performed when significant main effects were obtained.

Results

Metabolic Characterization

HF and CON Mice

Body weight increased (41% versus 18%; Figure 1A), gonadal fat pad mass was greater (0.91 ± 0.25 versus 0.35 ± 0.08 g), and fasting glucose (122 ± 7 mg/dL), area under the glucose tolerance test (GTT) curve (42,397 ± 3,064 versus 28,178 ± 1,507; Figure 1B), insulin (Figure 1C), and FFAs were higher (Figure 1D) in HF versus CON mice, respectively. Dual energy X-ray absorption indicated increased body and fat mass and decreased lean mass in HF versus CON mice, respectively (Figure I in the online data supplement). Dihydroethidium staining\[^{21}\] revealed no differences in superoxide anion (O\(_2^-\)) production (relative fluorescence intensity [RFI] units) in the endothelium (1438 ± 182 and 1255 ± 124) or vascular smooth muscle (904 ± 89 and 814 ± 143) of HF and CON mice, respectively (n=5 per group). Vascular NADPH oxidase activity was similar between HF and CON mice (not shown). The fold change in eNOS (0.99 ± 0.19) and ET-1 mRNA (1.41 ± 0.14; P=0.08) in HF relative to WT mice was not different (n=5 per group).

TTr-IR\(^{-/-}\) and WT Mice

IRs were absent and IGF1R mRNA was present in the vasculature of TTr-IR\(^{-/-}\) mice (Figure 2A; Online Figure II, A). Body weight (Figure 2B), gonadal fat pad mass (0.35 ± 0.08 and 0.22 ± 0.04 g, P=0.07), fasting glucose (Figure 2C), FFAs (2.0 ± 0.1 and 2.3 ± 0.1 mmol/L), and area under the GTT curve (17,486 ± 370 and 17,646 ± 1310; Figure 2C) were similar between WT and TTr-IR\(^{-/-}\) mice, respectively. Insulin was greater in both the fasting condition (ie, minute 0) and 30 minutes following IP glucose during the GTT in TTr-IR\(^{-/-}\) mice (Figure 2D). Vascular dihydroethidium staining was similar between TTr-IR\(^{-/-}\) and WT mice in the endothelium (1334 ± 97 and 1538 ± 88) and vascular smooth muscle (876 ± 26 versus 1025 ± 45; n=5 per group), respectively. The fold change in eNOS (0.98 ± 0.28) and ET-1 (1.06 ± 0.16) mRNA relative to WT mice was not different between groups (n=5 per group).

Because metabolic disturbances and vascular complications (see below) were observed in HF but not TTr-IR\(^{-/-}\) mice, we placed TTr-IR\(^{-/-}\) mice on HF chow (HF-TTr-IR\(^{-/-}\) mice). However, HF-TTr-IR\(^{-/-}\) mice did not develop systemic metabolic disturbances (Online Figure III, A through D) or arterial dysfunction (data not shown). As such, no further experiments were performed on these animals. Akt1\(^{-/-}\) mice have a metabolic profile similar to their WT littermates.\[^{14,15}\]

Vascular Signal Transduction

HF and CON Mice

Compared to vehicle, insulin increased the ratio of phosphor-
ylated (p)-ERK1/2 to total ERK similarly in aortae from CON and HF mice (Figure 3A). Insulin increased the ratio of p-Akt S473 and p-Akt T308 to total Akt in CON and HF animals, but insulin-mediated Akt phosphorylation at both residues was modestly yet significantly blunted in arteries from HF versus CON mice (Figure 3B). Furthermore, insulin increased the ratio of p-eNOS S1177 to total eNOS in arteries from CON but not HF mice (Figure 3C). Importantly, basal levels of p-eNOS S1177 were virtually nonexistent in arteries from HF versus CON mice, and insulin was not capable of increasing p-eNOS at this site. HF feeding did not alter vascular eNOS at T495, S617, or S635, or the upstream
kinases AMP-activated protein kinase (AMPK) or protein kinase (PK)A (Online Table I).

**TTr-IR**/−/− and WT Mice
Compared to vehicle stimulation, insulin increased the ratio of p-ERK1/2 to total ERK (Figure 4A), p-Akt S473 or p-Akt T308 to total Akt (Figure 4B), and p-eNOS S1177 to total eNOS in aortae from WT mice (Figure 4C). As expected, these effects were not observed in arteries from TTr-IR**/−/−** animals.

**Akt1**/−/− and WT Mice
Western blot experiments confirmed the absence of Akt1 protein in the vasculature of Akt1**/−/−** mice (Online Figure II, B). In aortae from WT and Akt1**/−/−** mice, insulin equivalently increased the ratio of p-ERK1/2 to total ERK1/2 (Figure 5A), p-Akt S473 or p-Akt T308 to total Akt (Figure 5B), and p-eNOS S1177 to total eNOS (Figure 5C). To explore whether insulin-mediated Akt phosphorylation in vessels from Akt1**/−/−** mice was mediated via the Akt2 isoform, Akt2 was immunoprecipitated from vascular homogenates obtained from Akt1**/−/−** and WT mice after stimulation with insulin or vehicle. Compared to vehicle, insulin increased the ratio of p-Akt S473 and p-Akt T308 to total Akt2 in both groups (Figure 5D). These results confirm insulin-mediated p-Akt in Akt1**/−/−** mice is mediated via the Akt2 isoform. Additionally, we sought to confirm the compartment wherein eNOS phosphorylation was occurring. Results in Online Figure IV show that insulin-stimulation evokes a robust increase in p-eNOS to total eNOS in intact arteries that is abolished in vessels denuded of endothelium. The pattern of insulin-mediated signaling for ERK, Akt, and eNOS among the 3 groups (ie, HF, TTr-IR**/−/−**, and Akt1**/−/−** mice and their respective controls) was similar between aorta and homogenates of iliac/femoral arteries.

**Blood Pressure**
Arterial blood pressure and heart rate were assessed in conscious mice for 30 seconds every 15 minutes over 72 hours starting 6 days following surgical implantation of a telemetry device in the abdomen. Blood pressure was greater
in HF versus CON mice (Figure 6A), but heart rate (646±45 versus 601±18 bpm, respectively) was similar between groups. Mean arterial pressure (Figure 6B) and heart rate (608±21 and 636±12 bpm) were similar between TTr-IR/−/− and WT mice, respectively. Likewise, arterial pressure (Figure 6C) and heart rate were similar between Akt1/−/− and WT mice (646±16 bpm) and WT mice (620±26).

Vascular Function

Because basal vascular eNOS phosphorylation was compromised, and hypertension existed, in HF but not TTr-IR/−/− or Akt1/−/− mice, we sought to determine whether functional indices of stimulated and basal eNOS activity displayed a similar pattern. Acetylcholine-evoked (Figure 7A) and N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA)-evoked (Figure 7B) eNOS activity and insulin-mediated vasorelaxation (Online Figure V, A) were blunted, whereas phenylephrine-induced vasocontraction was greater (Online Figure VI, A) in femoral arteries from HF versus CON mice. Relaxation to sodium nitroprusside (Figure 7C) was similar between groups. Acetylcholine-evoked (Figure 7D) and L-NMMA–evoked (Figure 7E) eNOS activity, as well as vascular smooth muscle responses to sodium nitroprusside (Figure 7F), were similar among femoral arteries between TTr-IR−/− and WT mice. Insulin-mediated vasorelaxation was precipitously absent in TTr-IR−/− versus WT mice only at the highest doses of phenylephrine (Online Figure VI, B). Results from Akt1−/− and WT mice (Figure 7G through 7I; Online Figure VI, C) were similar to those obtained from TTr-IR−/− mice. Results using aortae (data not shown) were similar to those obtained from femoral arteries. Vessel characteristics for femoral arteries are shown in Online Table II.

Treatment of BAECs and Arteries With Palmitate

Arterial dysfunction existed in HF mice, yet insulin-mediated signal transduction to eNOS via Akt1 in the vasculature was intact. Thus, we sought to determine whether a component of the systemic environment ie, elevated FFAs, was responsible for arterial dysfunction and lower basal and insulin-stimulated p-eNOS S1177 to total eNOS in the vasculature of HF versus CON mice. First, BAECs treated with 500 \textmu mol/L palmitate for 6 hours had lower basal and insulin-stimulated p-eNOS S1177, yet signaling to Akt and ERK was intact (Figure 8A through 8C). Second, 6 hours of palmitate treatment abolished insulin-mediated NO production by BAECs (Figure 8D). Finally, 3 hours of palmitate treatment abolished endothelium-dependent (Figure 8E) but not endothelium-independent vasorelaxation (not shown). In parallel experiments, neither endothelium-dependent nor endothelium-independent vasorelaxation was impaired in arteries treated for 3 hours with vehicle (BSA) or volume (milliQ water; data not shown). Basal p-eNOS was lowered to the same degree by 3
We assessed the contribution from vascular insulin signaling to eNOS in regulating endothelial function and blood pressure in mice with diet-induced obesity and in mice with complete (ie, TTr-IR−/− mice) and selective (ie, Akt1−/− mice) “resistance” to insulin signaling. Five main findings were observed. First, in HF mice, insulin-stimulated phosphorylation of eNOS was abolished, hypertension existed, but insulin-mediated signaling in the vasculature to ERK1/2 and Akt was preserved. Second, in TTr-IR−/− mice, insulin-stimulated phosphorylation of ERK1/2, Akt, and eNOS in the vasculature was predictably absent, but arterial blood pressure was similar to WT littermates. Third, the absence of Akt1 expression did not influence insulin-mediated phosphorylation of ERK1/2, Akt, and eNOS in the vasculature or blood pressure. Fourth, basal eNOS phosphorylation was almost absent in the vasculature of HF mice, and these animals displayed endothelial dysfunction together with systemic disturbances associated with diet-induced obesity. In contrast, basal eNOS phosphorylation, vascular function, and metabolic characteristics were similar among TTr-IR−/− and Akt1−/− animals and their respective WT littermates. These results suggested that hypertension and vascular dysfunction in mice with diet-induced obesity might be precipitated by reduced basal vascular eNOS phosphorylation caused by a component(s) of the circulating metabolic environment rather than by defective signaling via Akt1 to eNOS. The component we focused on was the elevation in FFAs observed in obese versus lean mice. In this regard, our fifth main finding was that basal and insulin-mediated eNOS phosphorylation and NO bioavailability are impaired, but signaling to Akt and ERK are intact, in endothelial cells incubated with the saturated FFA palmitate. Thus, elevated FFAs might be responsible for lowering vascular eNOS to a degree that precipitates cardiovascular complications in mice with diet-induced obesity.

Although impaired insulin signaling in the vasculature is widely accepted to be associated with obesity, type 2 diabetes, and generalized insulin resistance,1 the specific contribution of vascular insulin-resistance to the pathogenesis of arterial dysfunction and hypertension has not been definitively proven. For example, insulin resistance in spontaneously hypertensive rats (SHR) was accompanied by impaired vascular PI3K-dependent NO production, enhanced mitogen-activated protein kinase (MAPK)-dependent ET-1 secretion, and endothelial dysfunction versus control (WKY) rats.8 In that report, mesenteric arterial function was restored in SHRs by ET-1A/B receptor blockade or by MAPK/ERK kinase inhibition. Whereas this study and others2,9 suggest that an imbalance in vascular insulin-mediated signaling via Akt to eNOS together with intact or exaggerated MAPK-dependent ET-1 production has the potential to disrupt vascular homeostasis, a recent publication challenged this concept. Specifically, insulin-stimulated Akt phosphorylation was normal in arteries from mice with diet-induced obesity and glucose intolerance, although eNOS structure was disrupted and endothelial dysfunction was present.10 These results suggest that a component of endothelial dysfunction might be independent of upstream modulation via Akt.

The contribution of insulin-mediated signal transduction in the vasculature to blood pressure regulation is also unclear. For instance, blood pressure measured via tail cuff was similar between VENIRKO and WT mice,3 whereas hypertension existed in IRS-1−/− mice versus their respective controls.22 Because VENIRKO mice model nonselective insulin resistance and IRS-1−/− mice model PI3K/Akt selective “resistance” to IR-mediated signaling, these findings support the hypothesis that imbalanced downstream signaling from the IR disrupts cardiovascular homeostasis.1,4,23,24 In contrast, arterial pressures were higher in mice wherein both signaling pathways downstream from the IR were thought to be compromised (ie, heterozygous IRKO mice) in a balanced manner.11

In an attempt to clarify this issue, we compared insulin signaling in the vasculature, endothelial function, and systemic blood pressure in mice with diet-induced obesity to mice with genetic ablation of insulin receptors in all vascular tissues (TTr-IR−/−) or mice with genetic ablation of Akt1
We hypothesized that HF feeding would selectively impair insulin-mediated signaling to eNOS via Akt in the vasculature, and this defect would lead to hypertension and endothelial dysfunction. Our results did not support this hypothesis. Instead, relative to arteries from lean mice, insulin-mediated Akt phosphorylation was preserved but insulin-stimulated eNOS phosphorylation at S1177 was abolished in vessels from obese animals. Furthermore, hypertension and endothelial dysfunction existed in obese versus lean mice. These data indicate the lack of insulin-mediated eNOS phosphorylation at S1177 in the vasculature, systemic hypertension, and vascular dysfunction that existed in HF mice did not result from deficient upstream activation of eNOS via Akt.

In addition to diet-induced obesity produced by HF feeding, we used 2 genetic models to test our overall hypothesis. First, we reasoned that if insulin-mediated signal transduction was absent, ie, using TTr-IR−/− mice, then there likely would be no net effect on blood pressure. Our findings support this hypothesis and confirm a previous study wherein similar blood pressures existed between VENIRKO and WT mice but contrast with results indicating that heterozygous IRKO mice are hypertensive relative to their WT littermates. In this latter study, vascular insulin-mediated eNOS phosphorylation was attenuated in aortae from heterozygous IRKO versus WT mice. Although these data suggest the heterozygous IRKO phenotype might have resulted from impaired insulin signaling to eNOS, upstream kinases responsible for activating this enzyme were not evaluated. As such, it is not possible to know whether an imbalance in insulin-mediated signal transduction existed in vessels from heterozygous IRKO versus WT mice.

Mice with targeted deletion of Akt1 were used as a second approach to test our overall hypothesis. We reasoned that if selective resistance to insulin-stimulated eNOS phosphorylation via Akt in the vasculature contributed to hypertension, then blood pressure should be elevated in Akt1−/− versus WT mice. Mice with specific deletion of Akt1 were chosen for several reasons. First, of the 3 major Akt isoforms (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ), only Akt1 and Akt2 are present in aortae and femoral arteries of mice. Second, evidence from mouse lung endothelial cells indicates that Akt1 is the isoform predominantly responsible for basal and VEGF-stimulated NO production. Finally, because Akt1 mice do not possess a diabetic phenotype, contributions from circulating metabolic factors to endothelial dysfunction or hypertension do not exist. Contrary to our hypothesis, blood pressures were not different between Akt1−/− and WT mice. Surprisingly, insulin-mediated Akt and eNOS phosphorylation were intact in both groups. Because Akt1 protein was verified to be absent in the same
vessels wherein these results were obtained, we explored the possibility and confirmed experimentally that insulin-mediated eNOS S1177 phosphorylation in the vasculature can occur via the Akt2 isoform.

Whereas we observed robust insulin-mediated eNOS S1177 phosphorylation in vessels from Akt1−/− and WT mice, Ackah et al showed that VEGF-stimulated NO production was impaired in mouse lung endothelial cells obtained from Akt1−/− versus WT mice.16 Our studies suggest that in endothelial cells and the vasculature, insulin and VEGF differ importantly in their ability to phosphorylate eNOS S1177 via Akt2. In our study and that of Ackah et al, the presence of Akt2 in endothelial cells of Akt1−/− mice was confirmed. Thus, whereas VEGF does not stimulate eNOS phosphorylation in the absence of Akt1 via residual Akt2 in mouse lung endothelial cells, we clearly show that insulin-mediated activation of Akt2 is sufficient to activate eNOS in the vasculature. We do not know the molecular basis for these differences, but spatial differences in subcellular localization of Akt1 versus Akt2 and components of the VEGF or insulin signaling pathways might play a role. We also considered the possibility that differences arose from the fact that we studied signaling in whole artery homogenates versus cultured endothelial cells, because vascular compartments other than the endothelium might have contributed to the eNOS phosphorylation we observed. We believe this is unlikely, however, because insulin-mediated eNOS S1177 phosphorylation in intact vessels was abolished in arteries denuded of their endothelium (Online Figure IV). Thus, the discrepancy between studies likely resides in differences in the signal transduction mechanisms for VEGF versus insulin.

Basal eNOS phosphorylation was virtually absent in vessels from HF versus control mice, whereas it was similar between TTr-IR−/− and Akt1−/− animals and their respective controls. Because phosphorylation of eNOS S1177 positively regulates eNOS activity,5,25–27 it is reasonable that NO bioavailability might have been compromised in vessels from HF mice to an extent that precipitated endothelial dysfunction and hypertension relative to TTr-IR−/− or Akt1−/− animals. This is supported by results from 3 protocols using isolated vessels. First, tension development in response to eNOS inhibition (a functional estimate of basal eNOS activity28) was less in aortae and femoral arteries from HF versus control mice. Second, phenylephrine-induced responses were greater in HF mice, suggesting that endogenous opposition to vasoconstriction from NO might be compromised. Finally, acetylcholine-evoked vasorelaxation (a functional estimate of stimulated eNOS activity) was blunted in vessels from HF mice.
versus control mice. Because these end points were generally similar between TTr-IR<sup>−/−</sup> and Akt1<sup>−/−</sup> mice and their WT littermates, it is reasonable to speculate that minimal basal eNOS phosphorylation at S1177 observed in vessels from HF mice might have precipitated endothelial dysfunction and hypertension. Collectively, the lack of basal arterial eNOS phosphorylation at S1177, rather than deficient signaling to eNOS via Akt1 in the vasculature, appears to contribute importantly to hypertension that exists in mice with diet-induced obesity. Further evidence supporting this statement is that p-eNOS S617 (another eNOS phosphorylation target downstream from Akt) was similar in vessels from CON and HF mice. In this regard, if defective signaling via Akt to eNOS contributed to minimal p-eNOS S1177, then p-eNOS S617 likely would have been lower.

Findings from studies similar to ours implicate hyperglycemia, defective signaling to eNOS via AMPK, and elevated FFAs as important contributors to impaired arterial eNOS activity, function, and/or phosphorylation. Hyperglycemia in our HF mice was much less severe than values reported by Molnar et al, which might have contributed to increased oxidative stress and disruption of eNOS protein dimers in the vasculature. Whereas Wu et al demonstrated lower arterial p-AMPK T172 to total AMPK and impaired p-eNOS S1177 to total eNOS in arteries from fat-fed versus lean mice and in cultured cells treated with saturated versus unsaturated fatty acids, we found no difference in arterial p-AMPK between vessels from HF and CON animals. Du et al reported that reduced aortic eNOS activity in mice fed high-fat chow could be normalized by treatment with an antilipolytic agent that decreased fatty acid release from adipose cells. Moreover, Edirisinghe et al observed impaired endothelial function in aortic segments incubated with palmitate for 60 minutes. Because we observed a >3-fold elevation of FFAs in HF versus CON mice, we explored this mechanism further. When BAECs were incubated with palmitate, basal and insulin-stimulated eNOS phosphorylation was impaired, but signaling to ERK and Akt was intact. The palmitate-induced reduction of eNOS phosphorylation was sufficient to impair insulin-mediated NO production by BAECs and endothelium-dependent but not vascular smooth muscle function of isolated arteries. Results in BAECs and vessels exposed to palmitate mimic those we observed in arteries from HF mice. Because the source of fat in the HF diet primarily was from lard, it is possible that metabolites of saturated fatty acids (eg, the sphingolipid ceramide) might accumulate in response to HF feeding and lower arterial NO bioavailability. Indeed, preliminary data from our laboratory indicate vascular ceramide increases ∼2-fold (P<0.05) in mice exposed to HF feeding for 10 to 14 weeks. Although further work is warranted to elucidate the precise molecular signal(s) whereby HF feeding precipitates hypertension and vascular dysfunction, results from the present study indicate these cardiovascular complications occur via mechanisms that are independent of defective IR-mediated signaling to eNOS via Akt1 in the vasculature and might be related to the accumulation of FFAs and subsequent impairment of vascular NO production.

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Disclosures
None.

References
11. Symons et al. Insulin and Akt Regulation of Blood Pressure


Contribution of Insulin and Akt1 Signaling to Endothelial Nitric Oxide Synthase in the Regulation of Endothelial Function and Blood Pressure

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SUPPLEMENTARY MATERIALS FOR

CONTRIBUTION OF INSULIN AND Akt1 SIGNALING TO eNOS IN THE REGULATION OF ENDOTHELIAL FUNCTION AND BLOOD PRESSURE

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6 figures + 2 tables

Materials and Methods

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Utah. Mice were maintained in a temperature-controlled barrier facility with a 12-hour light/dark cycle and were given free access to food and water. 10-week old C57Bl6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and consumed standard rodent chow (D12450B, Research Diets, Inc; NJ) containing (kcal) 10% fat, 70% carbohydrate, and 20% protein (control diet; CON) during a one-week quarantine period. Next, mice either continued the CON diet or were placed on high fat chow (D12451, Research Diets, Inc; NJ) containing (kcal) 45% fat, 35% carbohydrate, and 20% protein for 10-12 weeks [high fat (HF) diet]. Subgroups of HF and CON mice were used for: metabolic characterization; vascular signal transduction; vascular function; detection of vascular superoxide anion generation; blood pressure; and RT-PCR. TTr-IR⁻/⁻ and WT mice were obtained from an on site colony developed using breeding pairs provided by Dr. D. Accili at Columbia University.¹ These mice were created by transgenically re-expressing the human IR in IR null mice using the transthyretin promoter which re-establishes IR expression in the liver, pancreatic islets and brains of TTr-IR⁻/⁻ mice, and is sufficient to prevent diabetic ketoacidosis that
causes early lethality in IR null mice.\textsuperscript{1-3} Subgroups of 20-25 week-old $TTr-IR^{+/}$ and WT mice were used for the same experiments as described for HF and CON mice. Akt1-/- and WT mice were obtained from an on site colony developed using breeding pairs provided by Dr. M. Birnbaum at the University of Pennsylvania.\textsuperscript{4, 5} The Akt1-/- mouse does not display a diabetic phenotype. Subgroups of Akt1-/- and WT mice were used to assess insulin signaling in the vasculature, vascular function (12-14 weeks old) and systemic arterial blood pressure (22-27 weeks old). Studies to assess: insulin signaling in the vasculature were performed on homogenates of aorta and iliac/femoral arteries; arterial function were performed using aorta and femoral arteries; superoxide anion content were performed on aorta and femoral arteries. Subgroups of $TTr-IR^{+/-}$ and WT mice that consumed diets identical to those described for CON and HF animals for 10-12 weeks were characterized metabolically and used to determine vascular function.

**Metabolic characterization.** Mice were fasted for 6 h by removal to a clean cage without food at the end of their dark (feeding) cycle i.e., 6 AM. After 6h mice were weighed, and blood glucose was determined (glucometer, Ascensia Elite XL) via a tail clip (i.e., 0-min). Next, glucose (1 mg/g, IP) was administered to conscious animals and blood glucose determined after 15, 30, 60, and 120 min. At 0 and/or 30-min, an extra volume of blood (~50ul) was obtained to assess serum insulin (ELISA, Linco, Billerica, MA) and free fatty acids (FFAs; colorimetric, half-micro test, Roche Diagnostics, Indianapolis, IN). Body composition was assessed using Dual Energy X-Ray Absorptiometry (DEXA; pDEXA Sabre Bone Densitometer, Norland Medical Systems, Fort Atkinson, WI).
**Signal transduction in the vasculature.** Jugular vein catheters were inserted into anesthetized mice (avertin, 0.15 mg/kg IP) using aseptic procedures. After 3 days of recovery and a 6 h fast, conscious mice were injected with vehicle (i.e., saline) or 3.8 mU insulin / g body weight. Within 5-min, mice were anesthetized, their chest was opened, and the aorta, iliac, and femoral arteries were bathed in ice-cold, oxygenated (95% O2 / 5% CO2), normal physiological saline solution (NPSS; pH 7.35-7.45) while adherent connective tissue was being removed from the respective vessels. Once isolated, vascular tissue from each mouse was placed in tubes containing 200 μL (aorta) or 150 μL (combination of right and left femoral and iliac arteries) ice-cold homogenization buffer [(0.05M HEPES, 0.01M sodium pyrophosphate, 0.01M sodium fluoride, 0.002M EDTA, 0.002M sodium orthovanadate, 1% Triton X-100, 10% Glycerol 100%, and 1:200 Sigma protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were sonicated and centrifuged for 15-min at 13,800 g at 4°C. Supernatant then was collected and protein concentrations determined [bicinchoninic acid (BCA) method] using a Thermo-max Microplate Reader (Molecular Devices, Menlo Park, CA) with bovine serum albumin (BSA) as a standard. Supernatants were stored at -80°C. Equal amounts of protein were suspended in loading buffer (60mM Tris-HCL, 25% Glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue or 2% SDS, 6.0 M Urea, 62.5 mM Tris-HCL, 160 mM DTT, and 0.001% bromophenol blue), incubated for 5-min at 95-100°C, and then resolved in a SDS-polyacrylamide gel (SDS-PAGE). Resolved proteins were transferred to a polyvinylidene difluoride (PDVF) membrane (Millipore Immobilon-P Transfer membrane; Millipore Corporation, Billerica, MA) at 4°C. Following transfer, the membranes were blocked with 5% nonfat dry milk in phosphate-
buffered saline with 0.1% Tween-20 (PBST) or 5% BSA in tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature. Blocked membranes were probed with primary antibodies for p-Akt S473, p-Akt T308, p-ERK, Akt 1, p-AMP-activated kinase (AMPK)α at T172 (p-AMPK T172), p-cyclic AMP response element binding protein (CREB) at S133 (p-CREB S133), p-eNOS S1177, p-eNOS S635, p-eNOS T495, (Cell Signaling, Danvers, MA) or p-eNOS S617 (Upstate Cell Signaling Solutions, Lake Placid, NY). Next, the membranes were washed with TBST, incubated with the appropriate secondary antibody conjugated to horseradish peroxidase [Anti-Rabbit IgG; Anti-mouse IgG (Amersham Biosciences, Piscataway, NJ), and visualized via ECL (Pierce detection kit, West Femto, Pierce Chemical Company, Rockford, IL). Membranes were stripped and reprobed with primary antibodies for total eNOS (BD Transduction Laboratories, Franklin Lakes, NJ), total Akt, total AMPKα, total CREB, (Cell Signaling), total ERK (Santa Cruz Biotechnology, Santa Cruz, CA), and/or α-tubulin (Sigma, St. Louis, MO) as appropriate. In Akt1-/- and WT mice Akt1 proteins were resolved on 10% SDS-PAGE, transferred and probed with specific antibodies (Cell Signaling). Detection was performed as described earlier. Membranes were stained with Coomassie blue (Bio-Rad Laboratories, Hercules, CA) for a loading control.

For Akt2 immunoprecipitation 500 µg of vascular homogenates from Akt1-/- mice were incubated for 3 hours at 4°C with anti-Akt2 antibodies (Cell Signaling). Next, protein A agarose beads (Upstate Cell Signaling Solutions, Temecula, CA) were added and incubated for 1 hour. Complexes were washed in lysis buffer and incubated at 95°C for 5 min. Protein was isolated by centrifugation at 8,000 rpm for 1 min, resolved on 10% SDS-PAGE, transferred and probed with anti-p-Akt S473 or anti-p-Akt T308
antibodies, and detected as described earlier. To normalize phosphorylated protein to total protein, blots were stripped and re-probed with total Akt2 antibody. Intensity (area times-density) of the immunoreactive bands in western blot and immunoprecipitation experiments was quantified using the Scion Image 1.62c program (Frederick, MD).

**Signal transduction in intact and denuded vessels.** 3.8 mU insulin / g body weight was administered intravenously to 10 conscious mice. Five-min later, animals were anesthetized, the chest was opened, and the entire aorta was excised. After sectioning the aorta into two segments, each was opened longitudinally. One segment was denuded of endothelium (- endothelium) using a modified cotton-tipped applicator, while the other was left intact (+ endothelium). Each segment was snap frozen in liquid nitrogen, and later sonicated, centrifuged, and aliquoted for subsequent protein and immunoblotting analyses. Identical procedures were performed in 10 mice that were administered vehicle (saline) intravenously. Therefore, four groups of vessels (n=10 each) were studied i.e., insulin-treated (+ and – endothelium) and vehicle-treated (+ and – endothelium). Immunoblot analyses of p-eNOS S1177 and total eNOS were performed as described earlier.

**Vascular function-**TTr-IR^+/−, Akt1^−/−, HF mice. Mice were anesthetized with 2-5% isoflurane. After opening the chest, segments of thoracic aorta were obtained just proximal to the bifurcation of the iliac arteries. Femoral arteries were isolated distal to the bifurcation of the internal iliac artery. During dissection, tissues were bathed in ice-cold, oxygenated NPSS. Once isolated and free of adherent tissue, arteries were
mounted on a wire-type myograph while immersed in a temperature-controlled, 8 ml tissue bath containing NPSS. After the arteries were mounted, the tissue bath was warmed gradually to 37°C over 30-min with vessels at 0 mg tension. Throughout each experiment, pH and temperature of all buffer solutions were checked at 20-30-min intervals, and contents of the tissue bath were exchanged at 15-min intervals.

Experimental protocols specific to each vessel type are described.

**Aortae.** When the tissue bath reached 37°C, tension on aortae was increased manually over 60 min to 1500 mg (i.e., 375 mg every 15-min). Final tensions did not differ among groups. Thirty-min later, receptor-mediated vasocontractile responses to phenylephrine (PE, 10^-8-10^-5 M) and potassium chloride (KCl, 10-100 mM) were assessed. Next, after arteries were precontracted to 65% of maximal PE-induced contraction and tension was stable, responses to: acetylcholine (ACh, 10^-8-10^-4 M; to determine endothelium-dependent vasorelaxation); insulin (50 - 5000 mU / ml); sodium nitroprusside (SNP, 10^-9-10^-4 M; to determine endothelium-independent vasorelaxation); and N^G monomethyl-L-arginine (L-NMMA, 10^-3 M; to estimate basal nitric oxide production) were performed. Each experimental protocol was separated by at least 30-min.

**Femoral arteries.** When the tissue bath reached 37°C, tension on femoral arteries was increased manually over 8-min to 200 mg (i.e., 50 mg every 2-min). Final tensions did not differ among groups (Online Table II). Thirty-min later, a series of internal circumference-active tension curves was constructed to determine the vessel diameter that evoked the greatest tension development (L_max) to 100 mM KCl. L_max tension did not differ among groups. The same protocols as described earlier for aortae were completed. For aortae and femoral arteries, two vessel segments per mouse were
treated identically and the results were averaged. All tension data were recorded continuously by a computer through an analog-to-digital interface card (Biopac Systems Inc., Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses.\textsuperscript{7-9}

**Vascular function - acute palmitate incubation.** Femoral arteries were obtained from 20-week old C57Bl6 mice to assess the effects of the saturated fatty acid palmitate (C16:0) on arterial function. Palmitate was used because it is the most physiologically abundant FFA. Mice were anesthetized and vessels were obtained and mounted as described. After determining $L_{\text{max}}$, performing a PE dose-response curve, and assessing endothelium-dependent vasorelaxation using acetylcholine, vessels were incubated with 500 uM palmitate, vehicle (1% BSA), or volume (400 ul milliQ water) for 3 h. Physiological FFA concentrations range from 250-500 uM, whereas pathophysiological concentrations can exceed 700 uM. After 3 hours, endothelium-dependent vasorelaxation and (45-min later) vascular smooth muscle function i.e., endothelium independent function, were assessed.

**Detection of vascular oxidant load.** Vascular indices of oxidant stress were assessed using the fluorescent dye dihydroethidium (DHE; Molecular Probes, Inc. Eugene, OR) and by measuring NADPH oxidase enzyme activity. DHE is a cell-permeant dye that emits blue fluorescence in the cytoplasm. In the presence of $O_2^-$, DHE is oxidized to ethidium which emits red fluorescence. Ethidium is a DNA-binding fluorophore that is impermeable to intact cell membranes. The degree of red fluorescence is proportional to the amount of intracellular $O_2^-$ that is present.\textsuperscript{10} Segments of thoracic aorta (∼1 mm,
6-9 segments per mouse) not used for function experiments were transferred to a microscope slide dish and treated for 30-min at 37°C in a light-protected, humidified chamber with either DHE (4 μmol/L concentration) or an equal volume of phosphate buffered saline (PBS; ~150 µl) to act as a vehicle / time control. Arterial segments then were rinsed once with 150 µl of PBS to remove unoxidized DHE. \( \text{O}_2^- \) production was estimated using confocal microscopy (Olympus Fluoview; Olympus America Inc., Melville, NY) as previously described.\(^8\) To assess whether changes in optics/acquisition occurred over time, low and high standards (Molecular Probes, Eugene, OR) of known fluorescence were quantified before and after each staining/imaging session. In previously published experiments\(^8\) we documented that: ethidium bromide is a DNA binding fluorophore; DHE is selective for \( \text{O}_2^- \) under similar experimental conditions; tissue fluorescence is enhanced in sections treated with dibromide monohydrate i.e., a positive control; and results obtained using NIH Image J software (Bethesda, MD) could be confirmed using another software program i.e., Volocity (Lexington, MA).\(^8\)

In another cohort of mice that consumed HF and CON diets, NADPH oxidase enzyme activity was assessed in entire aortic segments. After the appropriate duration on the respective diets, mice were anesthetized, the chest was opened, and the aorta was dissected free of adherent tissue. Next, vessels were snap-frozen in liquid nitrogen, and stored until all arteries could be assayed on the same day. After pulverizing tissue using a cindered glass-to-glass instrument, tissue was homogenized in Krebs solution. 5uM N, N-dimethyl-9, 9-biacridinium dinitrate (lucigenin, Sigma, St. Louis, MO) was then added to the vessel homogenate in the presence or absence (buffer blank) of 100 uM NADPH while luminescence was measured every 1.8 s for 60 s. Enzyme activity
(arbitrary units) was calculated as the sum value of luminescence during 60s. Luminescence from the buffer blank was subtracted from values + 100 uM NADPH. These values then were normalized initially for ug/L protein, and normalized finally to the Con group. Protein concentrations were determined using a commercially available BCA kit.¹¹

**Blood pressure.** Mice were anesthetized using 2-5% isoflurane. Using aseptic techniques, the abdomen was opened and a transmitter probe (Data Sciences International, St. Paul, MN)¹² was inserted and secured in place to allow ambulation with minimal hindrance. A catheter attached to the transmitter probe then was advanced subcutaneously and inserted into the right common carotid artery. Thirty second averages of blood pressure (systolic, diastolic, mean) and heart rate (HR) were recorded every 15-min, for three 24-hour periods, starting 6 days post surgery. Three 24-hour periods (i.e., days 7-9 post surgery) were averaged into one 24 hour period and used for data analysis.¹³

**Quantitative RT-PCR.** Segments of aortae were isolated from adherent tissue and placed immediately in RNA-Later (200 µl, Ambion, Austin, TX), stored at 4°C overnight, frozen in liquid nitrogen, and transferred to -80°C until homogenized in 1 ml TRlzol reagent (Invitrogen, Carlsbad, CA). RNA was isolated using the RNeasy MINI RNA isolation kit (Qiagen, Valencia, CA). The resulting RNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer.¹³ Total RNA (~300 ng) then was reverse transcribed into cDNA using Superscript III (Invitrogen). The resulting cDNA was combined with Platinum Taq DNA polymerase (Invitrogen), primers,
and SYBR-green (Invitrogen) fluorescent dye and transferred to a 96-well plate in triplicate. Amplification and detection of the fluorescence generated by the accumulating SYBR-green was performed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following cycle profile: 1 cycle at 95°C for 10 min, 40 cycles of 95°C for 15 sec, 59°C for 15 sec, 72°C for 30 sec, and 78°C for 10 sec, 1 cycle of 95°C for 15 sec, 1 cycle of 60°C for 15 sec, and 1 cycle of 95°C for 15 sec. Expression of each gene was normalized to the housekeeping gene i.e., cyclophylin (peptidylprolyl isomerase A, NM008907). Results from each experimental group i.e. HF or TTr-IR−/−, were compared to the respective control group (i.e., CON or WT) – which was assigned a value of 1.0. Primers were designed using Net Primer as follows: IR, forward primer 5'-TGG TCC TTT GGG AAA TCA CT-3' and reverse primer 5'-ATC CTT GAG CAG GTT GAC GA-3'; IGF1R, forward primer 5'-TCA GGC TAC CTC CCT CTC TG-3' reverse primer 5'-GCC CAA CCT GCT GTT ATT TC-3' ; ET-1, forward primer 5'-TCC AAG AAA GGA AAA CCC TGT-3' and reverse primer 5'-TTG TGC GTC AAC TTC TGG TC-3'; eNOS, forward primer 5'-GAC CCT CAC CGC TAC AAC AT-3' and reverse primer 5'-GCT CAT TTT CCA GGT GCT GTC TC-3'; and cyclophylin, forward primer 5'-AGC ACT GGA GAG AAA GGA TTT GG-3' and reverse primer 5'-TCT TCT TGC TGG TCT TGC CAT T-3' .

**Cell culture experiments.** Bovine aortic endothelial cells (BAECs; Cell Applications, CA) were grown in Dulbecco’s modified Eagles’s medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37°C. When BAECs were 70-80% confluent, DMEM was replaced with 1% bovine serum albumin (BSA) and
500 μM of the saturated FFA palmitate (C16:0). Palmitate was coupled to fatty acid-free BSA in the ratio of 2 mol palmitate to 1 mol of albumin. Vehicle controls were treated with equal concentrations of palmitate-free albumin. After 6 h incubation with palmitate or vehicle, BAECs were treated for 10-min with 100 nM insulin or vehicle. After insulin (or vehicle) stimulation, BAECs were collected as appropriate to determine: 1) total protein using the BCA protein assay kit (Thermo Scientific, Rockford, IL); 2) basal and insulin-stimulated p-Akt S473, p-Akt T308, p-ERK 1/2, p-eNOS S1177, and the respective total protein expression using immunoblotting procedures already described; 3) basal and insulin-stimulated NO production\textsuperscript{14, 15} and 4) cell viability in response to all treatments.\textsuperscript{16} p-eNOS to total eNOS was also assessed in BAECs incubated for 3 h in 500 μM palmitate or vehicle.

**Nitrate/Nitrite [NO\textsubscript{x}] in BAECs.** BAECs were incubated with 500 μM palmitate or vehicle for 6 h. For the last hour, growth media was replaced and supplemented with media containing 10 μM arginine with or without 100 nM insulin to stimulate NO production. Preliminary time-course experiments (0 - 90 min) indicated that 60-min was required to detect a robust increase in insulin-mediated NO production, and that this increase could be abolished by 100 mM L-NMMA (data not shown). 60-min after insulin or vehicle-treatment (i.e., at 6 h) 250 μL of cell media was obtained. NO that is produced in the media is rapidly oxidized to nitrite and then nitrate. When the nitrate-containing sample is combined with 0.5 M TRIS-HCl in the presence of a reducing wire, reduction of nitrate in the sample back to nitrite is facilitated. Nitrite then is reduced further to NO in an acidic iodide bath wherein it is detected by a commercially available
amperometric sensor (amiNO 700 Innovative Technologies, Sarasota FL). The sensor detects the electric current that is generated as NO diffuses from the sample solution to the selectively permeable surface of the probe. The electric current generated is directly proportional to the concentration of NO in the sample. A calibration curve using 25, 50, and 100 nM of nitrite standard was generated before and after each experiment, and used to determine the concentration of NO produced by BAECs.

**Cell Viability.** To exclude the potential contribution from cell death to the effects of palmitate exposure, BAECs were cultured and treated as described. After the respective treatments, culture dishes were rinsed 5 x to remove dead (i.e., non-adherent) cells. Next, 0.2 mL of 0.5% crystal violet dye was added to each well for 15-min to stain the DNA of remaining cells. After rinsing 5x with PBS, cells were detached from each well by adding 110 µL of 2% SDS to gently rocking culture dishes for 15-min. The percentage concentration of viable cells then was determined by measuring optical density of the resulting sample at 595 nm wavelength using an absorbance plate reader. Positive (trypsin) and negative (DMEM) controls were performed as appropriate. Cell viability for each treatment was determined relative to optical density represented by the positive and negative controls.
**Supplementary online figures (6) and tables (2)**

**Online Figure I.** Body composition of CON and HF mice was assessed using DEXA. Body weight and fat mass were greater while lean mass was less in HF vs. Con mice. Data are mean ± standard error of the mean from 10-12 mice per group. * p<0.05 HF vs. CON.
Online Figure II. Immunoblot analyses showing the absence of: insulin receptor (IR) protein in aortae from $TTr$-$IR^{+/−}$ vs. WT mice (A); and Akt1 protein in Akt1 null (Akt1−/−) vs. wild type mice. The loading controls i.e., $α$-tubulin and coomassie blue were not different between groups.
Online Figure III. Body composition of \(TTr-IR^{-/+}\) mice that consumed standard or high-fat chow (HF- \(TTr-IR^{-/+}\)) was assessed using DEXA (A). Lean mass was less and fat mass was greater in HF- \(TTr-IR^{-/+}\) vs. \(TTr-IR^{-/+}\) mice. *p<0.05 \(TTr-IR^{-/+}\) vs. HF-\(TTr-IR^{-/+}\). Blood glucose during the GTT (B), area under the GTT curve (C), and insulin before (0-min) and at 30-min of the GTT (D) were similar between groups. * p<0.05, 30-min vs. 0-min. Triglycerides (mg/ml) were not different between \(TTr-IR^{-/+}\) (0.9±0.1) and HF-\(TTr-IR^{-/+}\) mice (1.3±0.2). Data are mean ± standard error of the mean from 7 mice per group.
Online Figure IV. Insulin evoked a robust increase in p-eNOS to total eNOS in intact (+) arteries, but this response was abolished in vessels denuded (-) of endothelium. Data are mean ± standard error of the mean of 10 vessel segments per group. *p<0.05 insulin vs. vehicle; **p<0.05 intact (+) vs. denuded (-) in each respective group.
Online Figure V. Percent (%) vasorelaxation to insulin in isolated femoral arteries from HF (A) and TTr-IR⁻/⁻ mice (B). For each mouse, results from two vessels were averaged and counted as one observation. Each panel represents the mean ± standard error of the mean from 9-11 mice per group. * p<0.05 HF versus CON, and TTr-IR⁻/⁻ versus WT; + p<0.05 versus previous dose within the same group.
Online Figure VI. Vasocontraction (mg tension development / µm vessel length) in response to cumulative doses of phenylephrine (PE) in isolated femoral arteries from HF mice (A), TTr-IR/- mice (B), and Akt1/- mice (C), versus their respective controls. For each mouse, results from two vessels were averaged and counted as one observation. Each panel represents the mean ± standard error of the mean from 11-13 (HF and TTr-IR/-) or 4-8 (Akt1/-) mice per group. *p<0.05 CON versus HF mice, WT versus TTr-IR/- mice.
### Online Table I - Additional kinases and eNOS phosphorylation sites

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<th>Kinase</th>
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<td><strong>intracellular signaling kinases</strong></td>
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<tr>
<td>p-AMPK T172 / total AMPK</td>
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<td>p-CREB S133 / total CREB</td>
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<td><strong>eNOS phosphorylation sites</strong></td>
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<tr>
<td>p-eNOS T495 / total eNOS</td>
<td>0.51±0.08</td>
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<td>p-eNOS S617 / total eNOS</td>
<td>0.63±0.01</td>
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<tr>
<td>p-eNOS S635 / total eNOS</td>
<td>0.86±0.06</td>
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AMPK, adenosine monophosphate activated protein kinase; CREB, cyclic AMP response element binding protein; S, serine; T, threonine; eNOS, endothelial nitric oxide synthase. There were no differences between groups.

### Online Table II - Vessel characteristics

<table>
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<th>Femoral artery</th>
<th>WT</th>
<th>TTr-IR&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>WT</th>
<th>Akt1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<th>HF</th>
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</table>

WT, wild type mice; TTr-IR<sup>−/−</sup>, mice with genetic ablation of insulin receptors in all tissues except for brain, pancreas, and liver; Akt1<sup>−/−</sup>, mice with genetic ablation of the Akt1 isoform in all tissues; CON, mice that consumed standard rodent chow; HF, mice that consumed high-fat rodent chow.
References


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