A Novel Mechanism for Vascular Insulin Resistance in Normotensive Young SHRs

Hypoadiponectinemia and Resultant APPL1 Downregulation

Wenjuan Xing, Wenjun Yan, Peilin Liu, Lele Ji, Youyou Li, Lu Sun, Ling Tao, Haifeng Zhang, Feng Gao

Abstract—Vascular insulin resistance contributes to elevated peripheral vascular resistance and subsequent hypertension. Clinical observation showed that lower plasma adiponectin concentration is significantly associated with hypertension. This study was aimed to determine whether hypoadiponectinemia induces vascular insulin resistance before systemic hypertension and the underlying mechanisms. Four-week-old young spontaneously hypertensive rats (ySHRs, normotensive) and adiponectin knockout (KO; APN−/−) mice were used to evaluate the role of hypoadiponectinemia in insulin-induced vasodilation of resistance vessels. ySHRs showed significant vascular insulin resistance as evidenced by the blunted vasorelaxation response to insulin in mesenteric arterioles compared with that of age-matched Wistar-Kyoto controls. Serum adiponectin and mesenteric arteriolar APPL1 (an adaptor protein that mediates adiponectin signaling) expression of ySHRs were significantly reduced. In addition, Akt and endothelial NO synthase phosphorylation and NO production in arterioles were markedly reduced, whereas extracellular signal-regulated protein kinases 1/2 (ERK1/2) phosphorylation and endothelin-1 secretion were augmented in ySHRs. APN−/− mice showed significantly decreased APPL1 expression and vasodilation evoked by insulin. More importantly, treatment of ySHRs in vivo with the globular domain of adiponectin for 1 week increased APPL1 expression and insulin-induced vasodilation, and restored the balance between insulin-stimulated endothelial vasodilator NO and vasoconstrictor endothelin-1. In cultured human umbilical vein endothelial cells, globular domain of adiponectin upregulated APPL1 expression. Suppression of APPL1 expression with small interfering RNA markedly blunted the globular domain of adiponectin-induced insulin sensitization as evidenced by reduced Akt/endothelial NO synthase and potentiated ERK1/2 phosphorylations. In conclusion, hypoadiponectinemia induces APPL1 downregulation in the resistance vessels, contributing to the development of vascular insulin resistance by differentially modulating the Akt/endothelial NO synthase/NO and ERK1/2/endothelin-1 pathways in vascular endothelium in normotensive ySHRs. (Hypertension. 2013;61:00-00.)

Key Words: APPL1 ▪ endothelium ▪ hypertension ▪ hypoadiponectinemia ▪ vascular insulin resistance

Prehypertension is very prevalent worldwide (30% of the adult population), and is often associated with other cardiovascular risk factors and independently increases the risk of hypertension and subsequent cardiovascular events. Lifestyle modifications or drug intervention that can delay progression from prehypertension to hypertension would be of value.¹⁻³ Vascular insulin resistance is a common pathophysiological change observed in hypertension as well as many other diabetic cardiovascular diseases. The insulin-signaling pathway via phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial NO synthase (eNOS) induces vasodilation by regulating production of NO from endothelium,⁴ whereas insulin also regulates secretion of the vasoconstrictor endothelin-1 (ET-1) through Ras, Raf-1, mitogen-activated protein kinase kinase 1/2 (MEK1/2), and mitogen-activated protein kinase pathway and consequently induces vasoconstriction. Thus, the net hemodynamic action of insulin is dependent on a balance between its vasodilator and vasoconstrictor effects. Vascular insulin resistance, characterized by imbalance between insulin-induced endothelial vasodilator NO and the vasoconstrictor ET-1, contributes to elevated peripheral vascular resistance and subsequent hypertension.⁵ Factors that shift the vasoconstrictor action to vasoconstrictor effect of insulin tend to be more effective in preventing hypertension and improving insulin sensitivity.⁶ Previous studies have demonstrated that young spontaneously hypertensive rats (ySHRs) without hypertension showed an impaired insulin signaling (PI3K/Akt/eNOS) in the vasculature, suggesting that vascular insulin resistance is possibly a
risk factor preceding phenotypic manifestation of hypertension. However, the mechanisms responsible for vascular insulin resistance at prehypertensive stage remain unclear.

Adiponectin is an adipokine secreted by adipose tissue and is normally present in plasma. Adiponectin is reported as a potent insulin enhancer linking adipose tissue and glucose metabolism. Hypoadiponectinemia is caused by interactions of genetic factors, such as single nucleotide polymorphism in the adiponectin gene and environmental factors causing obesity. Clinical observation has revealed that hypoadiponectinemia is significantly associated with hypertension, suggesting that reduced adiponectin is one of the independent risk factors for hypertension. Moreover, hypoadiponectinemia is a marker for predisposition to hypertension in men, and also a predictor for the development of hypertension. However, the relationship between plasma adiponectin levels and vascular insulin resistance before hypertension has never been identified.

APPL1 is the first identified adaptor protein that interacts directly with adiponectin receptors (AdipoR1 and AdipoR2) by the phosphotyrosine-binding domain. Adiponectin induces adenosine monophosphate–activated protein kinase (AMPK) activation by promoting APPL1-dependent LKB1 cytosolic translocation in muscle cells. APPL1 also functions in insulin signaling through interacting with Akt and mediates GLUT4 translocation in muscle and adipose tissues. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is considered to be a key negative regulator of the PI3K/Akt pathway. LKB1-dependent upregulation of PTEN attenuates insulin signaling in endothelial cells. However, the role of APPL1 in the relationship between plasma adiponectin levels and vascular insulin resistance, and the underlying mechanisms in hypertension remains elusive.

Therefore, we hypothesized that hypoadiponectinemia may induce vascular insulin resistance in normotensive ySHRs, which is attributable to APPL1 downregulation in the resistance vessels. This study was aimed to evaluate whether and how hypoadiponectinemia influences insulin-induced vasodilation of resistance vessels using normotensive ySHRs and adiponectin KO (APN−/−) mice.

Methods
Please see the online-only Data Supplement for the detailed Methods.

Results
Vasorelaxation to Insulin Was Reduced in Mesenteric Artery Segments From Both Adult and Young SHRs

Similar to our previous report, young 4-week SHRs (ySHRs) had no significant difference in body weight, systolic blood pressure (SBP; Figure 1A), or systemic insulin sensitivity as assessed by QUICKI (QUICKI = 1/[log(insulin)+log(glucose)]; Figure 1B) when compared with their age-matched Wistar-Kyoto (WKY) counterparts (4-week WKY, yWKY). Both sodium nitroprusside (SNP, an endothelium-independent vasodilator) and acetylcholine (ACH, an endothelium-dependent vasodilator) stimulated vasodilation of mesenteric arterioles were comparable between ySHRs and yWKY rats (Figure S1A and S1B in the online-only Data Supplement). Importantly, as shown in Figure 1C, the ability of insulin to cause dose-dependent vasorelaxation was significantly impaired in vascular segments from ySHRs than those from yWKY rats (21.6±3.2% versus 41.6±4.4% to 10−6 mol/L insulin; P<0.05), suggesting an impaired insulin sensitivity in resistance vessels from ySHRs whose blood pressure had not been increased.

We further investigated the signaling pathway related to this local insulin resistance in ySHRs. The insulin-induced relaxation was abolished either by the removal of the endothelium or pretreatment with Nω-nitro-L-arginine methyl ester (L-NAME) and wortmannin, in preparations from both ySHRs and yWKY rats. Pretreatment with PD98059 and BQ123 significantly increased vasodilation induced by insulin in ySHRs. However, PD98059 preincubation could not increase insulin-induced vasodilation in yWKY rats (data not shown).

The Reduced Insulin–Mediated Activation of Akt/eNOS and Increased Activation of Extracellular Signal-regulated Protein Kinases 1/2 Were Accompanied With Decreased Adiponectin Levels, Expressions of AdipoRs and APPL1 in ySHRs
As shown in Figure 2A and 2B, serum adiponectin concentration and adiponectin mRNA level in adipose tissue from ySHRs were both significantly lower than that in yWKY rats. The transcript (Figure 2C and 2D) and protein levels (Figure 2F) of AdipoR1 and AdipoR2 in mesenteric arteries were decreased in ySHRs. Furthermore, APPL1 transcription and protein levels in mesenteric arteries were downregulated in ySHRs.

Figure 1. The vasorelaxation effect of insulin was impaired in normotensive young spontaneously hypertensive rats (ySHRs). A, Systolic blood pressures of ySHRs and yWKY rats. B, Quantitative insulin sensitivity check index in ySHRs and yWKY rats. C, Dose–response curves for insulin-induced relaxation were obtained from mesenteric arteries of ySHRs and yWKY rats. Data are expressed as percentage of the contraction to phenylephrine (PE). All values are presented as mean±SEM. *P<0.05; **P<0.01 vs yWKY (n=6–8).
Adiponectin Replenishment Increased Insulin-Stimulated Vasodilation in ySHRs

Treatment with recombinant human globular domain of adiponectin (gAd) had no significant effects on insulin’s vasorelaxation (44.2±5.4% versus 41.6±4.4% to 10^{-6} mol/L insulin; P>0.05) and APPL1 expression (P>0.05) in yWKY rats. However, after 1 week of gAd administration, insulin-induced vasorelaxation was partially restored in vascular segments from ySHRs (32.5±2.5% versus 21.6±3.2% to 10^{-4} mol/L insulin; P<0.05; Figure 3A), whereas SNP- and ACh-induced vasorelaxations changed insignificantly (data not shown). Western blotting analysis revealed that gAd treatment markedly increased APPL1 expression, consistent with APPL1 mRNA level detected in mesenteric arteries, although it did not change AdipoRs (Figure 3B and 3C). No significant difference in expressions of Akt, eNOS, and ERK1/2 was observed among all groups after gAd treatment, but insulin-induced phosphorylations of Akt and eNOS were markedly improved (10^{-7} mol/L; 30 minutes), and phosphorylation of ERK1/2 was reduced (Figure 3C). Notably, mRNA level of insulin-stimulated ET-1 was significantly decreased in gAd-treated ySHRs (Figure 3D).

Adiponectin Deficiency Resulted in Endothelial Dysfunction and Vascular Insulin Resistance

To substantiate the causative role of adiponectin deficiency in vascular insulin resistance, homozygous adiponectin KO (APN^{-/-}) mice were used whose serum adiponectin and adiponectin mRNA level in adipose tissue were undetectable (Figure S4A and S4B). There was no difference in the SBP between APN^{-/-} and wild-type (WT) mice (Figure 4A). Consistent with previous observations,^{13} APN KO mice showed more severely impaired glucose tolerance after 4 weeks of high-fat/high-sucrose diet (Figure S4C). SNP-stimulated vasodilation of the mesenteric artery segments was comparable among groups (Figure S4D). However, compared with WT mice, vasodilator responses to ACh and insulin were significantly reduced in the mesenteric arteries from APN^{-/-} mice (Figure S4E; Figure 4B). Heterozygous adiponectin KO (APN^{+/+}) mice whose serum adiponectin concentration and adiponectin mRNA level in adipose tissue were significantly reduced also showed a reduced endothelial function and impaired sensitivity of insulin in inducing vasorelaxation, although to a lesser extent than
that seen in APN−/− animals. Importantly, APPL1 mRNA and protein levels in the mesenteric arteries were all decreased (Figure 4C). In addition, insulin-induced phosphorylations of eNOS and Akt were both significantly reduced and insulin-stimulated ERK1/2 phosphorylation was significantly increased in arteries of APN−/− mice compared with WT (Figure 4D). Interestingly, although gAd treatment for 1 week did not change the expressions of AdipoR1 and R2, it did upregulate APPL1 expression and subsequently reverse the insulin-stimulated signaling and vasodilation in mesenteric arteries of APN−/− mice (Figure S5).

Adiponectin–APPL1–AMPK Signaling Exerted Opposite Effects on Insulin-Induced Akt/eNOS and ERK1/2 Signaling Cascades in Cultured Human Umbilical Vein Endothelial Cells

To further investigate the role of adiponectin in switching insulin’s actions from vasoconstriction to vasodilation, we used small interfering RNA (siRNA) targeting AdipoR1 and AdipoR2 to reduce their expression in human umbilical vein endothelial cells (HUVECs). As shown in Figure S6A and S6B, AdipoR1 and AdipoR2 expressions were significantly decreased by siRNA targeting AdipoR1 and AdipoR2, but...
not by scrambled siRNA. Treatment with gAd increased APPL1 expression in a dose- and time-dependent fashion to a maximum of 2.5-fold at 10 μg/mL gAd for 48-hour incubation (Figure 5A and 5B) in HUVECs with scrambled siRNA, but not siRNA of AdipoR1 and AdipoR2.

To directly clarify whether gAd enhances vasodilator effect of insulin via APPL1, siRNA targeting APPL1 was used to suppress APPL1 expression in HUVECs. APPL1 expression was significantly decreased by siRNA of APPL1, whereas its endogenous expression was not changed by scrambled siRNA sequence (Figure S6C and S6D). Incubation with gAd significantly upregulated APPL1, cytoplasm LKB1 levels, and AMPK activation; reduced PTEN expression (Figure S7); thus increased insulin-induced Akt/eNOS phosphorylations, and decreased phosphorylation of ERK1/2 (Figure 5C). This was accompanied by increased NO release and decreased ET-1 level in the culture medium (Figure 5D and 5E). Suppression of APPL1 expression mediated by siRNA in HUVECs significantly blocked its downstream LKB1 translocation from nuclear to cytoplasm, changed AMPK activation, PTEN expression, and consequently inhibited the insulin-sensitizing effects induced by gAd (Figure S7).

**Discussion**

The major findings from this study are as follows. First, hypoadiponectinemia induces vascular insulin resistance in normotensive ySHRs. Second, low-APPL1 expression is at least one of the factors mediating hypoadiponectinemia-induced shift of insulin signaling toward vasoconstriction in resistance vessels of ySHRs. Third, replenishment of exogenous adiponectin enhances the vasoactive action of insulin via upregulating APPL1 expression. Our findings demonstrate for the first time that hypoadiponectinemia and the resultant downregulation of APPL1 expression contribute to vascular insulin resistance in the normotensive ySHRs.

Clinical data showed that healthy individuals with prehypertension had significantly lower adiponectin plasma levels compared with healthy normotensives, and normotensive people with higher adiponectin show significant trends toward lower SBP and diastolic blood pressure. Both our laboratory and Schwartz’s laboratory have found that vascular insulin resistance, which is characterized by blunted insulin–induced vasorelaxation or imbalance of insulin signaling, occurs before systemic insulin resistance in hypertensive and high-fat diet models. However, as an insulin-sensitizing factor, how adiponectin affects vascular insulin resistance in prehypertension was not clear. In the present study, we found that vasodilator effect of insulin on mesenteric arteries was significantly impaired, although systemic insulin sensitivity remained normal in ySHRs. In addition, our data also suggest that it was reduced vascular relaxant reactivity to insulin, but not endothelial dysfunction (impaired ACh-induced vasorelaxation), that was responsible...
for the vascular insulin resistance in ySHRs. Interestingly, these animals also showed significantly reduced serum adiponectin levels. Consistent with this, adiponectin-deficient (APN−/− and APN+/−) mice exhibited impaired insulin-induced vasorelaxation. Moreover, treatment of ySHRs or APN−/− mice with exogenous adiponectin for one week partly restored vasodilatory effect of insulin (Figure 3; Figure S5). Maeda et al reported that hypoadiponectinemia was sufficient for the development of systemic insulin resistance in diet-induced obese mice. Our data showed that adiponectin deficiency aggravated vascular insulin resistance under pathological condition induced by high-fat/high-sucrose diet. Taken together, these results suggest that vascular insulin resistance was attributable to hypoadiponectinemia in prehypertensive SHRs.

There is insufficient evidence as to whether hypoadiponectinemia is causally related to hypertension. We observed that there was no significant difference in the SBP between APN−/− and WT mice, which was consistent with previous studies. Nevertheless, adiponectin deficiency was reported to accelerate high-salt intake–induced hypertension in mice and that hypoadiponectinemia contributed to the development of hypertension in obese KK-Ay mice, at least in part, in addition to its effect via systemic insulin resistance. Our finding that vascular insulin resistance of mesenteric arterioles was induced by hypoadiponectinemia is of particular importance in attempting to understand the causes of hypertension, because small arteries, rather than conduit artery, contribute substantially to vascular resistance, which plays a key role in the development of hypertension. This is supported by the report that by augment of adiponectin level, short-term calorie restriction improved vasodilatory function and reduced remodeling of resistant vessels, preventing an increase in blood pressure in SHRs. Taken together, hypoadiponectinemia, per se, is not sufficient for the development of hypertension, but may contribute to or accelerate the development of hypertension under pathological conditions, such as genetic predisposition or diabetes mellitus.

APPL1 mediates the signaling of adiponectin and its effects on metabolism by interacting directly with adiponectin receptors. Moreover, overexpression of wild-type APPL1 enhances basal GLUT4 translocation, whereas overexpression of dominant negative APPL1 inhibits basal, insulin- and adiponectin-stimulated GLUT4 translocation, suggesting that APPL1 also acts as a mediator of insulin-signaling pathway and plays a critical role in adiponectin-dependent insulin sensitization in skeletal muscle. In this study, we found that both the ySHRs and adiponectin KO mice showed reduced APPL1 expression accompanied with adiponectin deficiency in mesenteric arteries, which were partly restored by 1-week adiponectin treatment. Interestingly, APPL1 expression was not changed in adipose tissue or skeletal muscle. In addition, gAd increased APPL1 expression in a time- and dose-dependent manner in HUVECs. These data provided direct evidence that hypoadiponectinemia induces downregulation of APPL1 expression in resistance vessels, although the underlying mechanisms remain unclear. Cheng et al showed that neither acute nor long-term treatment of HUVECs with high concentrations of glucose and insulin had any effect on APPL1 expression, indicating that hyperinsulinemia and hyperglycemia may not be direct contributors to the decreased APPL1 expression in small mesenteric arteries. Here, we observed the decreased expressions of AdipoRs in ySHR, which may be attributable to the increased transcription factor FOXO1 expression and activation (Figure S3). Moreover, knockdown of AdipoR1 and AdipoR2 by siRNA blocked gAd-afforded increase of APPL1 expression in HUVECs, suggesting that hypoadiponectinemia may downregulate APPL1 expression in a receptor-dependent fashion in the resistance vessels.

Vascular actions of insulin play an important role in maintaining both hemodynamic and metabolic homeostasis under healthy conditions. In hypertensive states, the selectively impaired PI3K/Akt/eNOS pathway and the augmented ERK1/2 signaling cascade in vascular endothelium lead to decreased NO availability and enhanced ET-1 production, thereby tilting the balance between the vasodilator and vasoconstrictor actions of insulin toward endothelial dysfunction and hypertension. It was reported recently that transgenic expression of APPL1 prevented age- and obesity-induced impairment in insulin-induced vasodilation and reversed obesity-induced augmentation in insulin-evoked ET-1–dependent vasoconstriction. Genetic disruption of APPL1 shifted the effects of insulin from vasodilation to vasoconstriction. Moreover, chronic exercise increases APPL1 expression, which mediates exercise-induced increase of insulin action in the liver of diet-induced obese mice. The results from the present study showed that in adiponectin-deficient mice, downregulation of APPL1 expression impaired insulin-induced vasodilation, whereas augmented insulin-induced vasoconstriction. In addition, adiponectin treatment partly restored APPL1 expression, and thus reversed the imbalance between insulin-induced signaling in ySHRs and APN−/− mice. Suppression of APPL1 expression with siRNA blocked adiponectin-induced insulin sensitization in HUVECs. Moreover, in APPL1 KO mice fed with or without high-fat diet, SBP was higher than age-matched WT littermates. These intriguing findings raise the possibility that reduced APPL1 expression is causally associated with hypoadiponectinemia-induced vascular insulin resistance in prehypertension.

As an intracellular adaptor protein interacting with AdipoRs and mediating adiponectin-activated AMPK, APPL1 is also recognized to play a role in insulin-signaling pathway, because it interacts with PI3K and Akt. Our results showed that potentiation of insulin-induced Akt/eNOS/NO signaling and vasodilatation by APPL1 is partly attributable to its ability to increase translocation of LKB1 from the nucleus to the cytoplasm and AMPK phosphorylation, and subsequently decrease PI3K inhibitor PTEN expression in the endothelium. In addition, APPL1 also potentiates insulin-stimulated Akt activation by competing with the Akt inhibitor Tribbles. In contrast to the changes in the Akt/eNOS signaling, siRNA-mediated knockdown of APPL1 expression in HUVECs resulted in a significant elevation of insulin-induced phosphorylation of ERK1/2 and ET-1 production. It was reported that AMPK activation resulted in drastic inhibitions of Ras, Raf-1, and ERK activation induced by insulin-like growth factor 1. Wang et al demonstrated that APPL1...
altered insulin-stimulated phosphorylation status of Raf-1, thus suppressing activation of downstream kinase ERK1/2 in obesity animal models. Moreover, decrease of ET-1 partly contributes to adiponectin overexpression–induced improvement of the endothelial function in the early stage of diabetic nephropathy. Taken together, APPL1 mediated hypoadiponectinemia-induced imbalance between insulin-evoked Akt/eNOS/NO signaling and ERK1/2/ET-1 pathway via AMPK phosphorylation.

Perspectives

Our findings demonstrate for the first time that hypoadiponectinemia induces low-APPL1 expression that contributes to vascular insulin resistance by differentially modulating the activation of Akt/eNOS/NO and ERK1/2/ET-1 in vascular endothelium in normotensive ySHRs (Figure 6). Therefore, restoration of endogenous adiponectin production, supplementation with exogenous adiponectin, and treatment targeting APPL1 may have potential therapeutic value in the prevention and alleviation of endothelial dysfunction and vascular insulin resistance.

Limitation

One limitation of this study is that we only gave the short-term adiponectin treatment and did not observe the long-term effect of adiponectin on blood pressure change in ySHRs. The results described here are mainly the acute effects of adiponectin, which reveals the relationship between hypoadiponectinemia and vascular insulin resistance at prehypertensive stage. Whether this improved vascular insulin sensitivity by adiponectin treatment may delay the development of hypertension or whether targeting adiponectin deficiency may ameliorate hypertension in diabetic patients remains to be determined.

Acknowledgments

We thank Dr Wenli Yan for her kind help on language editing and proofreading of this article.

Sources of Funding

This study was supported by the State Key Program of National Natural Science Foundation of China (No. 81030005), National Basic Research Program of China (No. 2013CB531204), and the grants from the National Natural Science Foundation of China (Nos. 81270330, 81270301, 81170186).

Disclosures

None.

References

What Is New?
We have made several novel observations in this study:
• First, hypoadiponectinemia induces vascular insulin resistance in normotensive young spontaneously hypertensive rats.
• Second, decreased APPL1 expression is responsible for hypoadiponectinemia-induced imbalance of insulin signaling in resistance vessels of normotensive young spontaneously hypertensive rats.
• Third, supplementation of exogenous adiponectin improves vasoactive effects of insulin via upregulating APPL1 expression.

What Is Relevant?
• These results suggest that restoration of the production of endogenous adiponectin, supplementation with exogenous adiponectin, and treatments targeting APPL1 may have attractive therapeutic value in prevention and alleviation of endothelial dysfunction and vascular insulin resistance.

Summary
To the best of our knowledge, this is the first report demonstrating that hypoadiponectinemia induces low-APPL1 expression, contributing to vascular insulin resistance by differentially modulating the activation states of Akt/endothelial NO synthase/NO and ERK1/2/endothelin-1 in vascular endothelium in normotensive young spontaneously hypertensive rats (Figure 6).
A Novel Mechanism for Vascular Insulin Resistance in Normotensive Young SHRs: Hypoadiponectinemia and Resultant APPL1 Downregulation

Wenjuan Xing, Wenjun Yan, Peilin Liu, Lele Ji, Youyou Li, Lu Sun, Ling Tao, Haifeng Zhang and Feng Gao

Hypertension. published online March 11, 2013; Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231 Copyright © 2013 American Heart Association, Inc. All rights reserved. Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2013/03/11/HYPERTENSIONAHA.111.00728

Data Supplement (unedited) at:
http://hyper.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

A Novel Mechanism for Vascular Insulin Resistance in Normotensive Young SHRs:
Hypoadiponectinemia and Resultant APPL1 Downregulation

Wenjuan Xing, Wenjun Yan, Peilin Liu, Lele Ji, Youyou Li, Lu Sun, Ling Tao, Haifeng Zhang, Feng Gao

From the Department of Physiology (W.X., W.Y., L.J., Y.L., F.G.), Department of Cardiology, Xijing Hospital (W.Y., P.L., L.S., L.T., F.G.), and Experiment Teaching Center (H.Z.), Fourth Military Medical University, Xi’an, China

Short title: Hypoadiponectin and vascular insulin resistance
Supplemental Methods

Reagents. Phenylephrine (PE), acetylcholine (ACh), sodium nitroprusside (SNP), insulin, Nω-nitro-L-arginine methyl ester (L-NAME), wortmannin (Wm), PD98059, BQ123, and dimethyl sulphoxide (DMSO) were purchased from Sigma, St. Louis, MO, USA. Stock solutions of each drug were prepared in distilled water except for PD98059 and wortmannin (dissolved in DMSO). None of the vehicles used (including DMSO) at the final dilutions induced any significant vascular effects (assessed by appropriate controls in preliminary studies).

Animals. All experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals, and were approved by the Fourth Military Medical University Committee on Animal Care. Male SHRs and their age- and sex-matched normotensive Wistar-Kyoto controls (WKY) were purchased from Vital River Laboratories (Beijing, China). Homozygous (APN−/−) and heterozygous adiponectin knockout (APN+/−) mice (background strain: C57BL/6) were described as previously. KO and sex-matched WT littermates at 8 weeks of age were fed a high-fat/high-sucrose diet (30% fat, 15% sucrose) for 4 weeks to induce systemic insulin resistance. Systolic blood pressure (SBP) was measured with a tail-cuff system (BP-98A, Softron).

Treatment with adiponectin. At the age of 3 weeks, SHRs were treated with vehicle (PBS, n=6), or recombinant human globular domain of adiponectin (gAd, 2 µg/g/day×7 days, n=6). Two hours after the last treatment, animals were sacrificed, and blood samples and the mesenteric arteries were extracted.

Measurement of insulin sensitivity. Fasting blood glucose and insulin levels were measured with the use of a blood glucose meter ((Lifescan, USA) and an RIA test kit (Peninsula Laboratories), respectively. We measured insulin sensitivity using the quantitative insulin sensitivity check index (QUICKI). QUICKI was calculated using the following formula:

\[
QUICKI = \frac{1}{\log(I_0) + \log(G_0)},
\]

where I₀ is fasting insulin (µU/ml), and G₀ is fasting glucose (mg/dl).

Intraperitoneal glucose tolerance test (IPGTT). After 16 hours of fast, conscious mice were challenged with a glucose load of 1.5g/kg, administered intraperitoneally. Tail blood was taken at the times 0 min, 15min, 60min and 120min after the glucose load. The blood glucose levels were determined using an OneTouch II glucose meter (Lifescan, USA).

Functional assessment of murine mesenteric arterioles. Mesenteric arterioles were isolated from rats and mice, and cut into 3–4 ring segments with 1 mm long. The arteriolar segments mounted in a temperature-controlled myograph (DMT 610M) were perfused with PSS (containing (mM): NaCl 119, NaHCO₃ 15, KCl 4.6, MgCl₂ 1.2, NaH₂PO₄ 1.2, CaCl₂ 1.5 and glucose 5.5) continuously gassed with a mixture of 95% O₂ and 5% CO₂ (pH 7.4). An optimal passive tension (~1.5 mN) was applied for 1 hour before the experiments were started. In certain preparations, the endothelium was removed by gentle mechanical abrasion. Mesenteric arteriolar segments were precontracted with 1 µmol/L PE. A dose-response curve was obtained by cumulative addition of ACh (10⁻¹⁰ to 10⁻⁵ mol/L), SNP (10⁻¹⁰ to 10⁻⁵ mol/L), and insulin (10⁻¹⁰ to 10⁻⁸ mol/L). Relaxation at each concentration was measured and expressed as the percentage of force generated in response to PE. In some experiments, insulin dose response curves were repeated after pretreatment with L-NAME (a specific NOS inhibitor, 100 µM, 30 min), wortmannin (a PI3K inhibitor, 100 nM, 30min), PD98059 (an ERK1/2 inhibitor, 10 µM; 30min),
or BQ123 (an ET-1 inhibitor, 1 µM; 30min).

**Determination of serum adiponectin concentrations.** Total serum adiponectin concentrations were determined with a rat adiponectin ELISA kit (Adipobioscience, Santa Clara, USA) and a mouse ELISA kit (R&D Systems, Minneapolis, MN) per the instructions of the manufacturers.

**Preparation of plasma membrane and nuclear protein extracts.** Mesenteric arterial tissue was homogenized in buffer A containing (in mmol/L, pH 7.0): 10 NaHCO₃, 5 NaN₃, and then centrifuged at 7000×g for 20 min. The pellet was resuspended in buffer B (10 mmol/L Tris-HCl, pH 7.4), and centrifuged at 200×g for 20 min. The supernatant was gently layered on top of a 20% (vol/vol) Percoll gradient in buffer C (in mmol/L: 255 sucrose, 10 Tris-HCl (pH 7.4), 2 EDTA) and centrifuged at 55,000×g for 1 hour. The band at density of 1.030 was aspirated and pelleted by centrifugation at 170,000×g for 1 hour and resuspended in buffer C as plasma membrane solution. Protein concentration of plasma membrane solution was determined with BCA protein assay. AdipoRs content in plasma membrane was determined by Western blotting. Nuclear and cytoplasmic extractions were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). LKB1 content in nuclear and cytoplasmic fractions were determined by Western blotting.

**Western blotting analysis.** Protein samples were separated by electrophoresis on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA and incubated overnight with the appropriate primary antibodies respectively [anti-APPL1, anti-phospho(p)-Akt (Ser 473), anti-Akt, anti-p-ERK1/2, anti-ERK1/2, anti-p-AMPK (Thr 172), anti-AMPK, anti-PTEN (Cell Signaling Technology), anti-p-eNOS (Ser 1177), anti-eNOS (BD Biosciences), anti-AdipoR1, anti-AdipoR2, anti-LKB1 (Abcam)], followed by incubation with the corresponding secondary antibodies. The blots were visualized with ECL-plus reagent. β-actin was used as the internal loading control.

**Real-time PCR.** Total RNA was extracted from flash-frozen tissue using TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1 µg RNA by using the reverse transcription reagent kit (DRR047A, TaKaRa). Expression analysis of the reported genes was performed by real-time PCR using a PCR detection kit (DRR081A, TaKaRa) and ABI 7500 Sequence Detection System. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primers for mRNA expression analysis by Real-time PCR are listed in Supplementary Table S1. The threshold cycle (ΔΔCt) method of comparing expression data was applied and the relative quantitative values were expressed as $2^{-\Delta\Delta Ct}$ using the formula:

$$2^{-\Delta\Delta Ct} = [(Ct \text{ gene of interest - Ct internal control)} \text{ sample A} - (Ct \text{ gene of interest - Ct internal control)} \text{ sample B}]$$

**Cell culture and small Interfering RNA (siRNA) transfection.** Human umbilical vein endothelial cells (HUVECs) were grown in endothelial cell basal medium containing 2% fetal bovine serum and endothelial growth supplements and were used between passages 5~8. This study was approved by our institutional review board. Informed consent was given by persons donating umbilical cords. For gene silencing assay, siRNA for AdipoR1, AdipoR2, and APPL1 mRNA were designed and purchased from GenePharma (ShangHai, China). The sequences of the oligos are indicated in supplementary Table S2. HUVECs were transfected with siRNA specific to human APPL1 or scramble control by Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instructions. At 48 hours after transfection, cells were serum-starved for 6 hours and treated with different concentrations of gAd for various periods as specified in each
To determine the insulin-induced signaling pathway, after incubation of 10mg/ml gAd for 48 hours, the medium was changed into ordinary culture medium and then the HUVECs were exposed to $10^{-7}$ mol/L insulin for 30 min. The efficiency of gene knockdown was detected by Western blot analysis at 48 hour after siRNA transfection.

**Quantification of NO, and ET-1 release in endothelial cells.** Total NO production in culture medium was determined by measuring the concentration of nitrite, a stable metabolite of NO, with a modified Griess reaction method as reported previously. Levels of ET-1 in the media were measured with a sensitive ELISA kit (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer’s instructions.

**Statistical analysis.** All values in the text, table, and figures are presented as means ± SEM of n independent experiments. Statistical significance was determined by Student t test or ANOVA, and post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significances. In all statistical comparisons, probabilities of 0.05 or less were considered to be statistically significant.
References


### Supplemental Data

**Table S1.** Primer sequences used for real-time PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat adiponecin</td>
<td>5'-GGAAACTTGTGCAGGTTGGATG-3’</td>
<td>5'-GGGTCACCCTTAGGACCAAGA-3’</td>
</tr>
<tr>
<td>Rat AdipoR1</td>
<td>5'-CTTCTACTGCTCCACACGC-3’</td>
<td>5'-TCCCAGGAACACTCTCTGCTC-3’</td>
</tr>
<tr>
<td>Rat AdipoR2</td>
<td>5'-CCACACAAACCAAAGATCCG-3’</td>
<td>5'-CCCTTCTCTTGGGAGAAATGG-3’</td>
</tr>
<tr>
<td>Rat APPL1</td>
<td>5'-ACCACACACAGACGAAACCAGC-3’</td>
<td>5'-TGTACGCTGCTCCTTGGACCA-3’</td>
</tr>
<tr>
<td>Rat ET-1</td>
<td>5'-ATGGATTATTTTCCCCAGTGAT-3’</td>
<td>5'-GGGAGTTGACCCAGATGA-3’</td>
</tr>
<tr>
<td>Rat GAPDH</td>
<td>5'-ACCACAGTCATGCCATCAC-3’</td>
<td>5'-TCCACCACCTGTTGCTGTA-3’</td>
</tr>
<tr>
<td>Mouse adiponecin</td>
<td>5'-AGGATGCTACTGTGGAAAGCTCTC-3’</td>
<td>5'-CAGTCACTTTGGGTATCAGGAGAG-3’</td>
</tr>
<tr>
<td>Mouse APPL1</td>
<td>5'-ATCAGGAGCTGCCTGGAA-3’</td>
<td>5'-GGCTCACAACCTTGAGAA-3’</td>
</tr>
<tr>
<td>Mouse GAPDH</td>
<td>5'-ACCCAGAAGATTGGGATGG-3’</td>
<td>5'-CACATTGGGAGGATGGACAC-3’</td>
</tr>
<tr>
<td>Human AdipoR1</td>
<td>5'-GGTTTGCCACTCCTAAGC-3’</td>
<td>5'-AGCATAAAGGCAGCTCCA-3’</td>
</tr>
<tr>
<td>Human AdipoR2</td>
<td>5'-ATGGCCAGCCTCTACAC-3’</td>
<td>5'-GCCGATCATGAAACGAACT-3’</td>
</tr>
<tr>
<td>Human APPL1</td>
<td>5'-UAGCAGAUAGUCGUGUCCUUAAGC-3’</td>
<td>5'-GCUAAGGGCAACGACUACUGCUA-3’</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>5'-TCGGAGTCAGGATTGTG-3’</td>
<td>5'-GCATCGCCCTGCATTGATT-3’</td>
</tr>
</tbody>
</table>
Table S2. Sequences of siRNA.

<table>
<thead>
<tr>
<th>Genes</th>
<th>sense</th>
<th>anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPL1 (h)</td>
<td>5'-UAGCAGAUAGCUUGUUGCCUUUAGC-3'</td>
<td>5'-GCUAAAGGACAACGACUAUCUGCUA-3'</td>
</tr>
<tr>
<td>AdipoR1 (h)</td>
<td>5'-AAUGGCUUCCAAACUCCGUUGG-3'</td>
<td>5'-CCACCAAGGAGAUUUGGAGCCAUU-3'</td>
</tr>
<tr>
<td>AdipoR2 (h)</td>
<td>5'-CACACCUAGCCUAAAACUTT-3'</td>
<td>5'-AGUUUGAGGUCAGGUGUGTT-3'</td>
</tr>
</tbody>
</table>
Figure S1: Both endothelium-independent and -dependent vasodilator effects of mesenteric arterioles were comparable between ySHRs and yWKY rats. A and B: Dose-response curves for SNP-(A) and ACh-induced (B) relaxation were obtained from mesenteric arteries of ySHRs and yWKY rats. Data are expressed as percentage of the contraction to PE. All values are presented as means ± SEM. (n= 6-8).
Figure S2: APPL1 expressions were not changed in adipose tissue or skeletal muscle from both ySHRs and yWKY rats. A and B: Representative Western blots showing APPL1 expression in adipose tissue (A) and skeletal muscle (B) in ySHRs and yWKY rats. (n=3).
**Figure S3:** phosphorylated and total FoxO1 expression in mesenteric arteries of ySHRs and yWKY rats. A: Representative Western blots showing the expressions and phosphorylations of FoxO1 at Ser256. B, C, and D: phosphorylated, total FoxO1 and ratio of phosphor-FoxO1 to total FoxO1 in mesenteric arteries. All values are presented as means ± SEM. *P < 0.05 vs. yWKY (n=3-4).
Figure S4: Adiponectin level, systemic insulin sensitivity and vascular responses of APN knockout mice. A and B: Serum adiponectin concentrations (A) and adiponectin mRNA levels in adipose tissue (B) of APN<sup>−/−</sup> and WT mice. C: The IPGTT was performed as described in Supplemental Methods, and blood glucose was measured at 0, 15, 60, and 120 min in WT and APN<sup>−/−</sup> mice fed with a high-fat/high-sucrose diet (30% fat, 15% sucrose) for 4 weeks. D and E: Dose-response curves for SNP- (D) and ACh-induced (E) relaxation were obtained from mesenteric arteries of APN<sup>−/−</sup>, APN<sup>+/−</sup> and WT mice. Data are expressed as percentage of the contraction to PE. All values are presented as means ± SEM. *P < 0.05 vs. WT; #P < 0.05 vs. APN<sup>+/−</sup> (n=4-6).
Figure S5: Treatment with gAd improved vasodilation to insulin and reversed the insulin signaling changes in mesenteric arteries of APN−/−. A: Dose-response curves for insulin-induced relaxation were obtained from mesenteric arteries of APN−/−. B: Representative Western blots showing the expressions of APPL1, Akt, eNOS, ERK1/2, and phosphorylations of Akt, eNOS, and ERK1/2. All values are presented as means ± SEM. *P < 0.05 vs. APN−/− (n=3-4).
Figure S6: HUVECs were transfected with siRNA specific for AdipoR1, AdipoR2, APPL1 or scrambled control. The mRNA levels and expressions of adipoRs (A and B) and APPL1 (C and D) were determined by real time-PCR and Western blotting. *$P < 0.05$, **$P < 0.01$ (n=5-6).
**Figure S7:** HUVECs were transfected with siRNA specific for APPL1 or scrambled control for 48 hours, followed by serum starvation for 6 hours and stimulation with or without gAd and/or insulin (10^{-7} mol/L). Representative Western blots showing APPL1, LKB1, PTEN, AMPK, and phosphorylation of AMPK at Thr172 (n=5-6).