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

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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:

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

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 10⁻⁶ 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^(-ΔΔCt) using the formula:

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

**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
figure legend. 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.

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<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Rat adiponectin</td>
<td>5’-GGAAACTTGTGCAGGTTGGATG-3’</td>
<td>5’-GGGTCACCCTTTAGGACCAAGAA-3’</td>
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<tr>
<td>Rat AdipoR1</td>
<td>5’-CTTCTACTGCTCCCACACGC-3’</td>
<td>5’-TCCCAGGAACACTCCTGCTTC-3’</td>
</tr>
<tr>
<td>Rat AdipoR2</td>
<td>5’-CCACACAACACAAAGAATCCG-3’</td>
<td>5’-CCCTTCTTCTTTGGGAGAATGG-3’</td>
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<tr>
<td>Rat APPL1</td>
<td>5’-ACCACAAACAGCACAGAACCACG-3’</td>
<td>5’-TGTACGCCCTGCCCTCTTGACCA-3’</td>
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<td>Rat ET-1</td>
<td>5’-ATGGATTATTTTCCCATGAT-3’</td>
<td>5’-GGGAGTTGTTGACCCAGATGA-3’</td>
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<td>Rat GAPDH</td>
<td>5’-ACCACAGTCCATGCCATCAC-3’</td>
<td>5’-TCCACCACCCCTGTGCTGTA-3’</td>
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<td>Mouse adiponectin</td>
<td>5’-AGGATGCTACTGGCAAGCTCTC-3’</td>
<td>5’-CAGTCAGTGGGTATCATGGTAGAG-3’</td>
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<td>Mouse APPL 1</td>
<td>5’-ATCAGGAGCTGCCTGGAA-3’</td>
<td>5’-GGCTCAACACCATGTTGGAA-3’</td>
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<td>Mouse GAPDH</td>
<td>5’-ACCCAGAAGCCTGGATGG-3’</td>
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<td>Human AdipoR1</td>
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<td>5’-GCUAAGGACAACGACUAUCUCUGCUA-3’</td>
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<tr>
<td>Human GAPDH</td>
<td>5’-TCGGAGTCAACGGATTTG-3’</td>
<td>5’-GCATCGCCCCACTTGATT-3’</td>
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Table S2. Sequences of siRNA.

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<th>Genes</th>
<th>sense</th>
<th>anti-sense</th>
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<td>APPL1 (h)</td>
<td>5’-UAGCAGAUAGCGUUGCUUUAGC-3’</td>
<td>5’-GCUAAGGACAACGACUAUCUGCUA-3’</td>
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<tr>
<td>AdipoR1 (h)</td>
<td>5’-AAUGGGCUCCAAACUCUUGUGG-3’</td>
<td>5’-CCACCAAGGAGAUUGGAGCCCAUU-3’</td>
</tr>
<tr>
<td>AdipoR2 (h)</td>
<td>5’-CACACCUGACCUACACUTT-3’</td>
<td>5’-AGUUUGAGGUCAGGUGUGTT-3’</td>
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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).