C1q/TNF-Related Proteins, A Family of Novel Adipokines, Induce Vascular Relaxation Through the Adiponectin Receptor-1/AMPK/eNOS/Nitric Oxide Signaling Pathway


Objective—Reduced plasma adiponectin (APN) in diabetic patients is associated with endothelial dysfunction. However, APN knockout animals manifest modest systemic dysfunction unless metabolically challenged. The protein family CTRPs (C1q/TNF-related proteins) has recently been identified as APN paralogs and some CTRP members share APN’s metabolic regulatory function. However, the vasoactive properties of CTRPs remain completely unknown.

Methods and Results—The vasoactivity of currently identified murine CTRP members was assessed in aortic vascular rings and underlying molecular mechanisms was elucidated in human umbilical vein endothelial cells. Of 8 CTRPs, CTRPs 3, 5, and 9 caused significant vasorelaxation. The vasoactive potency of CTRP9 exceeded that of APN (3-fold) and is endothelium-dependent and nitric oxide (NO)-mediated. Mechanistically, CTRP9 increased AMPK/Akt/eNOS phosphorylation and increased NO production. AMPK knockdown completely blocked CTRP9-induced Akt/eNOS phosphorylation and NO production. Akt knockdown had no significant effect on CTRP9-induced AMPK phosphorylation, but blocked eNOS phosphorylation and NO production. Adiponectin receptor 1, but not receptor 2, knockdown blocked CTRP9-induced AMPK/Akt/eNOS phosphorylation and NO production. Finally, preincubating vascular rings with an AMPK-inhibitor abolished CTRP9-induced vasorelaxative effects.

Conclusion—We have provided the first evidence that CTRP9 is a novel vasorelaxative adipokine that may exert vasculoprotective effects via the adiponectin receptor 1/AMPK/eNOS dependent/NO mediated signaling pathway.

Key Words: diabetes mellitus ■ endothelial function ■ nitric oxide ■ signal transduction

Endothelial dysfunction, characterized by impaired endothelium-dependent vasodilatation, is a common feature across major cardiovascular diseases, and precedes more severe pathology.1 Conduit arterial endothelial dysfunction is a well-established antecedent of hypertension and atherosclerosis, whereas dysfunction of peripheral (arteriolar and capillary level) endothelium contributes to insulin resistance and metabolic syndrome pathogenesis.2 In both animal models and humans, restoration of endothelium-dependent vasodilatation has been shown to be a requisite therapeutic intervention for vascular health.

Adiponectin (APN), an insulin-sensitizing adipokine predominantly secreted by adipocytes, possesses potent protective effects against endothelial dysfunction.3 The positive association between serum APN level and intact endothelium-dependent vasorelaxation has been demonstrated repeatedly.4–6 Moreover, reduced plasma APN in diabetic patients has been demonstrated to be associated with endothelial dysfunction. APN knockout mice have been generated and studied by many groups. When metabolically challenged (eg, high-fat diet), APN-null mice develop insulin resistance, endothelial dysfunction and vascular injury; however, in the absence of dietary or metabolic stress, these animals show a relatively modest phenotype.7–9 These results suggest that potent compensatory mechanisms are in place.

Recently, a highly conserved family of APN paralogs, designated C1q tumor necrosis factor (TNF) related proteins (CTRPCs), has been discovered. Most of the known members (CTRPCs 1 through 10) consist of 4 distinct domains, including an N-terminal signal peptide, a short variable domain, a collagen-like domain, and a C-terminal C1q-like globular domain.10 11 Both CTRPs and APN belong to the C1q/TNF

Received on: February 11, 2011; final version accepted on: August 2, 2011.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.111.231050
protein superfamily, which continues to grow as more C1Q domain proteins are discovered.\textsuperscript{12} Investigated for its structural similarity to APN, the CTRP family has been demonstrated to have substantial metabolic effects similar to APN, particularly CTRP1 and 3.\textsuperscript{12,13} Accordingly, it has recently been proposed that CTRPs, particularly CTRP1 and 3, may have partially overlapping metabolic function effectively compensating for APN-deficient disease states.\textsuperscript{14,15} However, whether members of the CTRP family possess potent vascular protective effects similar to APN and could thus compensate for APN in its absence or dearth, remains completely unknown.

Therefore, the aims of this study were (1) to determine whether any members of the CTRP family exert vasorelaxative effects; (2) to compare the vasorelaxative potency of different CTRP types against APN; and (3) to elucidate the responsible underlying molecular signaling pathways.

**Materials and Methods**

All experiments in this study were performed with adherence to the NIH Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care. Detailed methods for construct and expression of CTRPs, assessment of vasorelaxation, culturing of endothelial cells, and determination of molecule signaling pathways were described in the online supplement (available at http://atvb.ahajournals.org).

**Statistical Analysis**

All values in the text and figures are presented as means±SD of n independent experiments. Data (except Western blot density) were subjected to t test (two groups) or one-way ANOVA (three or more groups) followed by Bonferroni correction for post hoc t test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn’s post test. Probabilities of 0.05 or less were considered statistically significant.

**Results**

**Select CTRP Subtypes Induced Vasorelaxation**

In aortic rings isolated from wild type C57BL/6 mice, ACh (an endothelium-dependent vasorelaxation agonist) induced a concentration-dependent vasorelaxation similar to that elicited by acidified NaNO\textsubscript{2} (an endothelium-independent vaso-dilator). At the maximal concentration studied (100 μmol/L), ACh and NaNO\textsubscript{2} caused 78±5\% and 80±4\% vasorelaxation, respectively, in aortic ring segments (Figure 1A), confirming intact endothelial and smooth muscle function. In preliminary experiments, as expected, aortic vascular rings denuded of endothelium exhibited no vasorelaxative response to ACh stimulation, while maintaining a normal dilatory response to acidified NaNO\textsubscript{2} (data not shown).

Administration of CTRP1, 2, 4, 6, or 7 caused neither significant vasoconstriction nor vasorelaxation (data not shown) and were not further investigated. However, administration of CTRP3, 5, or 9 caused significant vasorelaxation in a concentration-dependent fashion. Of the 3 vasoactive CTRPs, CTRP3 and 5 exhibited vasorelaxative effect tanta-mount to APN (Figure 1B). CTRP9 demonstrated greatest vasorelaxative potency, exceeding that of APN. Specifically, CTRP9 (3 μg/mL) administration elicited significant vasorelaxation comparable to that caused by 10 μg/mL APN. At the maximal concentration observed (ie, 10 μg/mL), CTRP induced 60±5\% vasorelaxation, whereas the same concentration of APN only caused 40±8\% vasorelaxation (P < 0.01) (Figure 1B). Based on this observed potency, CTRP9 was used in further experiments and for mechanistic dissection.

**CTRP9-Induced Vasorelaxation Is Endothelium-Dependent and NO Mediated**

To determine whether CTRP9 induces vasorelaxation by directly acting on smooth muscle cells or indirectly via stimulating vasorelaxative molecule production by endothelial cells, the aortic vascular endothelial layer was gently denuded as previously described.\textsuperscript{16} As summarized in Figure 1C, CTRP9 administration failed to cause vasorelaxation, indicating that CTRP9 acts as an endothelium-dependent vasorelaxative agent. Finally, we determined whether NO is the endothelium-generated molecule that is responsible for mediating the vasorelaxative effects of CTRP9. Aortic vascular rings were pretreated with L-NAME (100 μmol/L) as summarized in Figure 1D, pretreatment with L-NAME completely blocked vascular vasorelaxative response to CTRP9.

**CTRP9-Stimulated NOX Production and Caused AMPK, Akt, and eNOS Phosphorylation in ECs**

Having demonstrated that CTRP9 induces vasorelaxative response in an endothelium-dependent and NO-mediated fashion, we further investigated the molecular signaling pathways responsible for CTRP9 vasorelaxation. Endothelial nitric-oxide synthase (eNOS) is the enzyme responsible for vascular physiological NO production; APN has been shown to activate eNOS via AMPK signaling.\textsuperscript{17,18} To determine whether CTRP9 induces endothelial cell NO production via AMPK signaling, HUVECs were incubated with CTRP9;
consequent NO production, AMPK phosphorylation (at Thr\(^{172}\) of its \(\alpha\) subunit), and eNOS phosphorylation (Ser\(^{1177}\)) were determined. As summarized in Figure 2, CTRP9 induced NO production in a dose-dependent (Figure 2A) and time-dependent (Figure 2B) manner. At 3 \(\mu\)g/mL, substantially increased NO production was observed within 15 minutes of CTRP9 administration, followed by modest increases tapering with time. Treatment with L-NAME abolished CTRP9-induced NO production enhancement. The experiments were also conducted using globular APN; CTRP9 exhibited nearly tripled potency compared to APN in eliciting NO production (Figure 2A). Moreover, CTRP9 administration had no effect on expression level of AMPK, Akt, and eNOS. However, CTRP9 enhanced the phosphorylation of AMPK, Akt, and eNOS in dose-dependent manner (Figure 2C, 2D, and 2E). Similar results were obtained in mouse aortic endothelial cells. mAEC incubation with CTRP9 (0.3, 1, and 3 \(\mu\)g/mL) for 15 minutes enhanced NO production (Supplemental Figure IA), AMPK phosphorylation (Supplemental Figure IB), and eNOS phosphorylation (data not shown) in concentration-dependent manner. Additionally, full length CTRP9, produced in human mammalian cells, similarly enhanced NO production (Supplemental Figure IC), eNOS phosphorylation (Supplemental Figure ID), and AMPK phosphorylation (data not shown) in concentration-dependent manner. Similar to APN, the globular isoform of CTRP9 demonstrated greater potency than its full-length isoform.

**Suppression of AMPK Expression By siRNA Blocks CTRP9-Induced Akt/eNOS Phosphorylation and NO Production in HUVECs**

The results presented in Figure 2 and Supplemental Figure I suggest that CTRP9 stimulates endothelial NO production by AMPK/Akt-mediated eNOS phosphorylation. To obtain direct evidence supporting a causative role of AMPK activation and CTRP9-induced NO production, additional experiments were performed. Forty-eight hours after transfection with
siRNA specifically targeting AMPK, HUVECs were incubated with CTRP9 (3 \mu g/mL) for 15 minutes. Western blot analysis confirmed siRNA suppressed 70% AMPK expression (Figure 3A), which completely blocked CTRP9-induced eNOS and Akt phosphorylation (Figure 3B and 3C) and abolished CTRP9-induced NO production (Figure 3D).

Suppression of Akt Expression By siRNA Had No Effect on AMPK Phosphorylation But Attenuated CTRP9-Induced eNOS Phosphorylation and NO Production

Previous studies have demonstrated that AMPK can cause eNOS phosphorylation either directly or indirectly via Akt activation.18 Because CTRP9 caused significant phosphorylation of both AMPK and Akt, we further determined the role of Akt in CTRP9-eNOS signaling. Forty-eight hours after transfection with siRNA specifically targeting Akt, HUVECs were incubated with CTRP9 (3 \mu g/mL) for 15 minutes. As summarized in Figure 4, suppression of Akt expression by siRNA (>70%, Figure 4A) had no effect on AMPK phosphorylation (Figure 4B), but significantly (although incompletely) blocked CTRP9-induced eNOS phosphorylation (Figure 4C) and NO production (Figure 4D). These data demonstrate that Akt is a downstream kinase of AMPK in CTRP9-induced eNOS phosphorylation and NO production.

Figure 3. CTRP9-induced eNOS activation and NO production in human umbilical vein endothelial cells (HUVECs) is AMPK-dependent. After AMPK siRNA knockdown, HUVECs were incubated with gCTRP9 (C1q tumor necrosis factor-related proteins; 3 \mu g/mL) for 15 minutes. A, Western blot analysis confirmed siRNA induced significant AMPK protein concentration reduction. AMPK knockdown significantly attenuated gCTRP9-induced Akt (B) and eNOS phosphorylation (C), and gCTRP9-induced NO production (D). n=5 to 6 repeated experiments/condition. **indicates \textit{P}<0.01.

Figure 4. The role of Akt in C1q tumor necrosis factor-related proteins (CTRP)-9–induced endothelial nitric-oxide synthase (eNOS) activation and NO production in HUVECs. After Akt siRNA knockdown, human umbilical vein endothelial cells (HUVECs) were incubated with gCTRP9 (3 \mu g/mL) for 15 minutes. A, Western blot analysis confirmed siRNA induced significant Akt protein concentration reduction. Akt knockdown had no significant effect on gCTRP9-induced AMPK phosphorylation (B) but significantly attenuated gCTRP9-induced eNOS phosphorylation (C) and gCTRP9-induced NO production (D). n=5 to 6 repeated experiments/condition. **indicates \textit{P}<0.01.

CTRP9 Activates AMPK in an AdipoR1-Dependent Fashion

Two APN receptors, AdipoR1 and AdipoR2, have been cloned,19 and several putative cell surface molecules, including T-cadherin and calreticulin/CD91, also bind with APN and mediate APN’s “ancient” collectin-like functions.20 However, whether CTRPs interact with any of these APN receptors/binding proteins and their role in mediating subsequent biological functions remains largely unknown. Currently, CTRP5 is the only CTRP member whose receptor dependency has been investigated. Experimental results suggest that CTRP5 exerts its metabolic effect in an AdipoR-independent fashion.21 To determine whether AdipoR1 and/or AdipoR2 are involved in CTRP9-mediated AMPK activation and NO production, we used siRNA to specifically downregulate endogenous AdipoR1 and AdipoR2 production. Forty-eight hours after transfection with siRNA specifically targeting AdipoR1 and AdipoR2, HUVECs were incubated with either vehicle or CTRP9 (3 \mu g/mL) for 15 minutes. Western blot analysis confirmed siRNA-induced suppression of AdipoR1 and AdipoR2 by 60% to 75% (Figure 5A). As illustrated in Figure 5B, downregulation of AdipoR1, but not AdipoR2, significantly reduced CTRP9-induced phosphorylation of AMPK, Akt, and eNOS, as well as NO production (Figure 5C) in HUVECs. Moreover, no significant difference in these outcome parameters was observed between AdipoR1 knockdown, and AdipoR1 and AdipoR2 double-knockdown cells.
We next determined whether there was direct interaction between AdipoR1 and CTRP9. We incubated HUVECs with CTRP9 and cross-linked with DTBP, and we used specific CTRP9 antibody to immunoprecipitate this protein alone with its binding partner. Indeed, AdipoR1 was coimmunoprecipitated in a complex with CTRP9 (Figure 5D), suggestive of a direct interaction involving CTRP9 binding to AdipoR1.

CTRP9 Accelerates Vascular Structure Formation In Vitro
In order to determine the downstream biological effects of increased eNOS activity and NO production, we investigated whether CTRP9 induced endothelial cell differentiation to form capillary-like structures when HUVECs were plated on Matrigel matrix. CTRP9 treatment promoted capillary-like tube formation (Figure 6A). These results suggest that CTRP9 promotes protective effects in endothelial cells.

CTRP9 Improved Endothelium-Dependent Vasorelaxation in Aortic Rings From HFD Mice
Consistent with previous results, concentration-dependent vasorelaxation in response to ACh was impaired in vascular segments isolated from mice fed a high-fat diet (Figure 6B). However, concentration-dependent vasorelaxation in response to NaNO2 remained unchanged in these vessels (Figure 6C). These results indicate that high-fat diet-induced hyperlipidemia caused significant endothelial dysfunction. Moreover, as Supplemental Figure IIB demonstrates, CTRP9 is clearly detected in circulating plasma using Western blotting method. More importantly, 3 weeks after high-fat
diet, CTRP9 levels fell to approximately 40% of normal. Supplementing exogenous fCTRP9 to the animals (1 μg/g body weight/day), a dose determined from a pilot study that restored plasma CTRP9 to normal level; see Supplemental Figure IIB) for 1 week via an osmotic pump improved endothelial function, as evidenced by significant improvement of the ACh dose-response curve (Figure 6B). Taken together, these results demonstrated that CTRP9 is present in circulation and that the observed decline in hyperlipidemic condition is pathologically relevant and significant.

**CTRP9-Induced Vasorelaxation Is Completely Blocked When AMPK Is Inhibited**

Our in vitro experimental results demonstrated that AMPK plays an essential role in CTRP9-induced endothelial NO production. In a final attempt to establish a causative role of AMPK in CTRP9-elicited vasorelaxation, we determined the effect of AMPK inhibition on CTRP9-induced vasorelaxative response. As summarized in Supplemental Figure IIA, pretreatment with Compound C, a selective AMPK inhibitor, completely blocked CTRP9-induced vasorelaxation.

**Discussion**

Although structurally related, the CTRP family members are functionally diverse. CTRP1 inhibits collagen-induced platelet aggregation by blocking the binding of von Willebrand factor to collagen.22 CTRP2 has been reported to induce AMPK phosphorylation, resulting in increased glycogen accumulation and fatty acid oxidation.23 Recombinant CTRP3, also known as CORT26/cardinucin of mainly chondrocyte origin, stimulates proliferation of chondrogenic precursor cells by activating extracellular-signal-regulated kinase 1/2 and Akt signaling pathways.24 Mutation of CTRP5, localized in the lateral and apical membranes of the retinal pigment epithelium and ciliary body, is associated with late-onset retinal degeneration.25 CTRP6 is requisite for African swine fever virus replication in HeLa and HT144 cells.26 Secreted from adipose tissue, CTRP9 reduces serum glucose in mice, and forms heterotrimers with APN.27 Despite ongoing investigations, information concerning both regulation and functional attributes of the CTRP family proteins remains unknown. Their elucidation will provide insight to both normal and disease states, as well as potential therapeutics treating the latter.

In this study, we demonstrated for the first time that CTRP3, 5, and 9 exert vasorelaxative effects, with CTRP9 being the most potent (possessing nearly 3 times the vasorelaxative effect of APN). CTRP9-mediated vasorelaxation is dependent on intact endothelium. Addition of L-NAME to solution bath blocked CTRP9-mediated aortic ring vasorelaxation. Together, these results clearly demonstrate that CTRP9 is an endothelium-dependent and NO-mediated novel vasorelaxative agent.

To dissect the underlying mechanisms responsible for CTRP9-induced vascular dilation, we used both in vitro HUVECs and mouse aortic endothelial cells and blocked various signaling molecules potentially involved in NO production. The present study provides the first direct evidence that CTRP9 increases endothelial NO production via the AdipoR1/AMPK/Akt/eNOS signaling pathway. eNOS is the enzyme responsible for physiological NO production in the vasculature. Direct phosphorylation of eNOS leads to its activation and NO production.28,29,30 AMPK, a central regulator of cellular energy metabolism and vascular reactivity modulation, stimulates eNOS by phosphorylating eNOS at Ser1177,31,32 Our results demonstrate that CTRP9 enhances Akt/eNOS phosphorylation and NO production in an AMPK-dependent fashion. Furthermore, the vasorelaxative effect of CTRP9 was abolished by Compound C, an AMPK inhibitor (Supplemental Figure IIA), further demonstrating that AMPK is the principal kinase responsible for CTRP9-induced eNOS phosphorylation, NO production, and subsequent vasorelaxation. In comparison to globular APN, CTRP9 had nearly tripled the potency in eliciting NO production in HUVECs (Figure 2A).

CTRP9 activated both AMPK and Akt (Figure 2C and D), each capable of phosphorylating eNOS.32,33 To determine the hierarchy of the roles of AMPK and Akt signaling in CTRP9-induced eNOS phosphorylation and NO production, we used siRNA knockdown of AMPK and Akt expression in HUVECs. Suppression of either AMPK or Akt expression resulted in a significant attenuation of CTRP9-induced eNOS phosphorylation and NO production, implicating both molecules' mechanistic involvement. Recent studies report eNOS regulation by APN-stimulated phosphorylation is complicated by potential AMPK-Akt cross-talk.18,34 However, we have obtained evidence that AMPK is the most upstream molecule in CTRP9-induced eNOS phosphorylation and NO production. Specifically, our results demonstrated that AMPK suppression blocked CTRP9-induced Akt phosphorylation at Ser473 (Figure 3B), but Akt suppression had no effect on CTRP9-induced AMPK phosphorylation at Thr172 (Figure 4B). CTRP9's downstream effects of eliciting increased eNOS activity and NO production resulted in accelerated capillary-like tube formation (Figure 6A). Furthermore, using an established high-fat-diet induced model of endothelial dysfunction, we demonstrated in vivo CTRP9 treatment for 1 week significantly enhanced endothelial function, evidenced by significantly improved dose-response curve to ACh (Figure 6B).

Both AdipoR1 and AdipoR2 are surface membrane proteins with 7 transmembrane domains, sharing similar molecular structure with each other, with expression detectable in most tissues and cell types. Whereas AdipoR1 is prevalent in the skeletal muscle and heart, AdipoR2 is the chief hepatic subtype.35–37 Aortic endothelial cells express both receptor types but predominantly AdipoR1.38 Previous to this study, whether CTRP9 interacts with 1, both, or neither APN receptor subtype was completely unknown. siRNA mediated knockdown of both AdipoR1 and R2 or AdipoR1 alone significantly attenuated CTRP9-induced AMPK/eNOS phosphorylation, resulting in marked HUVEC NO production reduction. Knockdown of AdipoR2 alone resulted in no significant effects. Additionally, via specific crosslink study, we demonstrated that AdipoR1 and CTRP9 directly interact (Figure 5D). These results provide the first direct evidence that AdipoR1 may function as a CTRP9 binding protein. Recently, studies on knockout mice indicate that the two
receptors mediate different signaling events in liver, with AdipoR1 primarily acting on AMPK, and AdipoR2 primarily affecting PPARα,26,39,40 consistent with our study’s mechanistic data. The adapter protein APPL1, containing a pleck-strain homology domain, a phosphotyrosine-binding domain, and a leucine zipper motif, appears to be a key intracellular effector of APN’s effects, via binding to the N-terminus of AdipoR1.41 Studies elucidating the intracellular signaling pathways of APN and CTRP are required and are currently ongoing.

In conclusion, we have demonstrated that CTRP subtypes 3, 5, and 9 exert vasorelaxative effects. Although CTRP3 and 5 share vasorelaxative potency tantamount to APN, the vasorelaxative effect of CTRP9 exceeds APN by nearly 3-fold. CTRP9 elicits vasorelaxation in an NO-dependent fashion. Finally, CTRP9 induces endothelial NO production in an AMPK-dependent manner, and AdipoR1 acts as a likely receptor of CTRP9 (Supplemental Figure IIIB). We have demonstrated that CTRP9 may contribute as an important regulator. Overlapping vasculoprotective function with APN and may have important therapeutic value in the treatment of endothelial dysfunction.

Limitation
Unlike APN, an adipokine that has attracted enormous attention and has been extensively investigated in the past decade, CTRPs have been cloned only very recently. Many fundamental questions, including their transcriptional regulation under physiological and pathological conditions, and their physiological plasma levels and pathological alterations remain completely unknown. Interestingly, our most recent preliminary experimental results suggested that adipocyte mRNA expression of multiple CTRPs is markedly reduced in high-fat diet induced type 2 diabetic animals. As such, our current novel finding that CTRP9 as an endothelium-dependent vasorelaxative agonist that exceeds the potency of APN is significant, and represents the first step toward our full understanding of the physiological and pathological importance of these novel adipocytokines.

Sources of Funding
This research was supported by the following grants: NIH HL-63828, HL-096686, the American Diabetes Association 7-08-RA-98 (X.-L.M.), NIH DK084171, and the American Heart Association SDG2260721 (G.W.W.). Dr Qijun Zheng is supported by a special training grant from Xijing Hospital, the Forth Military Medical University, China. Jonathan M. Peterson is supported by an NIH National Research Service Award (F32DK084607).

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2011;31:2616-2623; originally published online August 11, 2011;
doi: 10.1161/ATVBAHA.111.231050

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Materials and Methods

Construct and expression of globular CTRPs:

All globular domains of mouse CTRP genes were generated by PCR and cloned into the prokaryotic protein expression vector pET45b (Novagen, Merck, USA), with exceptions of CTRP 8 and 10 (Whereas the gene encoding CTRP 8 is present in humans and other select vertebrate species, it is absent in mice and rats. CTRP 10 is highly conserved through evolution, but has not yet been cloned, and was not tested in this study.). All constructs were verified by DNA sequencing. Globular CTRPs prokaryotic expression vector was transferred into BL21(DE3) bacterium protein expression host, grown in LB medium and shaken overnight at 37°C. Protein expression inducer IPTG was added to medium (final concentration 1mM). The solution was shaken for 4-5 hours, and subject to 5,000 RPM centrifugation. Proteins were purified in native condition by Ni-NTA resin per manufacturer’s instructions (Novagen, #70666-3, Merck, USA). Endotoxin was removed by endotoxin-removing column (Sterogene ActiCleanEtoc resin, Carlsbad, CA), desalted, and concentrated by centrifugation (Millipore Centricon, Plus-20). Purified proteins were stored at -70°C until use.

Aortic ring preparation and apparatus for assessment of vasorelaxation:

Vessel chamber experiments were performed as previously described. Briefly, Adult male wild type C57BL/6 mice were anesthetized with 3% isoflurane, and descending aortic segments were isolated. Vascular segments were placed into ice-cold Krebs–Henseleit (K–H) buffer consisting of (in mM): NaCl 118, KCl 4.75, CaCl2.2H20 2.54,
KH2PO4 1.19, MgSO4·7H2O 1.19, NaHCO3 25, and glucose 10.0. Aortic segments were carefully cleaned of fat and loose connective tissue, and sectioned into rings of 2–3 mm length, which were mounted upon stainless steel hooks and aerated at 37°C, in a 95% O2/5% CO2 mixture. Suspended in 5 ml of K–H tissue buffer, the aortic rings were connected to FORT-10 force transducers (WPI, Sarasota, FL) capable of recording vasculature constriction/dilation via a MacLab data acquisition system. The rings were stretched to an optimum preload of 0.8 g force (determined in preliminary experiments), and allowed to equilibrate for 60 minutes. During this period, the K–H buffer in the tissue bath was replaced every 15 minutes, and vascular ring tension was adjusted until 0.8 g preload force was maintained. After equilibration, the rings exposed to 100 nM of U-46619 (9,11-epoxymethano-PGH2, Biomol Research Laboratories, Plymouth Meeting, PA) to ensure stabilization of the vascular smooth muscle (maximally effective concentration determined in preliminary experiments). After agonist washout, ring re-equilibration was permitted. Twenty minutes after the initial washing, 50nM of U-46619 was added to each ring bath to generate approximately 0.8 g vascular contraction. Once stable contraction was achieved, acetylcholine (ACh), a vasorelaxive agent eliciting endothelial NO production, was added to the bath in cumulative concentrations of $10^{-8}$–$10^{-4}$ M to evaluate endothelial vasorelaxive function. After ring response to cumulative ACh stabilized, bath washout, and ring re-equilibration to baseline occurred. The procedure was then repeated with $10^{-8}$–$10^{-4}$ M dose increments of acidified NaNO2 (prepared by dissolving NaNO2 in 0.1 N HCl titrated to pH 2.0), an agent eliciting vasorelaxation in endothelium-independent manner. Rings exhibiting relaxation less than 60% in response to maximal ACh concentrations were excluded from further study.
After achievement of stable vasorelaxation by ACh and acidified NaNO₂ (as described above), repeat solution bath washout and ring re-equilibration to baseline was permitted. Each of the different CTRP family members (including CTRP1–7 and CTRP9) were evaluated separately, and added in cumulative concentrations of 0.3, 1, 3, and 10 µg/ml. APN was tested in similar manner as a positive control. To evaluate the involvement of NO in CTRP’s observed vasorelaxive effects, Nω-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of NO synthesis (dose 100 µmol/L), was also tested in conjunction with CTRPs manifesting significant vasorelaxive action.

**Cell culture:**

Human umbilical vein endothelial cells (HUVECs, Cell Applications, San Diego, CA) were cultured in endothelial basal medium-2 containing 10% fetal bovine serum (FBS) and premixed endothelial cell growth supplements per manufacturer’s instructions. Cells were seeded at 7×10⁵ per well in 2ml DMEM in a six-well dish. All cells in the experiment were used within 4–7 passages, at 80–90% confluence, and were examined under microscopy to ensure demonstration of specific immunohistochemical and morphologic characteristics of endothelial cells. Prior to experimental treatments, the cells were made quiescent by replacing the growth medium with human endothelial-SFM basal growth medium (Gibco), containing 5 mmol/l glucose and no serum or growth factor supplementation. HUVECs were serum-starved for 3 hours in all experiments before CTRP addition.

Mouse aortic endothelial cells (mAECs, cAP-m0001,Angio-Proteomie, Boston MA 02215) were isolated from Balb/C mouse aortae. Cells were cultured in DMEM medium containing 20% FBS, and utilized in studies within 6-10 passages.
**siRNA-mediated knockdown of signaling proteins**

siRNA duplex oligonucleotides were designed from human cDNAs encoding AMP kinase-α1 (AMPK-α1), Akt1, adiponectin receptor-1 (AdipoR1) and receptor-2 (AdipoR2). Four pooled AMPK-α1, Akt1, AdipoR1, or AdipoR2 specific siRNA duplexes were used to respectively knockdown AMPK-α1, Akt1, AdipoR1, and AdipoR2 expression per manufacturer’s instructions (Millipore/Upstate), with inclusion of a non-silencing siRNA control. The siRNA sequences targeting AMPK-α1 were 5’-UGCCAUACACUAUCAAUAdTdT-3’(sense) and 5’-UAUUAUGAGAGGUAGGCAdTdT-3’(antisense), the siRNA sequences targeting AdipoR1 were 5’-GGA CAACGACUAUCUGCACATT-3’ (sense) and 5’-TGTAGCAGATAGTCGGTTGTCCTT-3’ (antisense), and the siRNA sequences targeting AdipoR2 were 5’-GGAGUUUCGUUUCAUGAGAUCGGTT-3’(sense) and 5’-CCGATCATGAAACGAAACTCCTT-3’(antisense). Among all Akt isoforms, Akt1 is the predominant and biologically active isoform expressed in endothelial cells. The siRNA sequences targeting Akt1 were from Cell Signaling, Akt siRNAII ID# 6510, which inhibits human or mouse Akt1 expression, but will not affect Akt2 or Akt3 expression. After achieving 80% confluency in six-well plates, HUVECs were transfected with siRNA (final concentration 50 nM).

**Total NO production measurement**

To detect endothelial NO production, HUVECs were cultured in 6-well plates, and experiments were performed 1 day after confluence. HUVECs were serum-starved for 3 hours, and then permitted 15 minute incubation with CTRP9. Resultant medium nitrite
(NO$_2^-$) and nitrate (NO$_3^-$) levels were determined by use of a chemiluminescence NO detector (Siever 280i NO Analyzer).

**Immunoblotting**

Tissue homogenate proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and Western blotted with monoclonal antibody against eNOS, and polyclonal antibody against the following proteins: Ser$^{1177}$ phosphorylated eNOS, Ser$^{473}$ phosphorylated Akt/Akt, Thr$^{172}$ phosphorylated AMPK/AMPK (all from Cell Signaling Technology, Danvers, MA, USA), and AdiopR1 and AdiopR2 (both from Abcam, Cambridge, MA). Nitrocellulose membranes were incubated with horseradish peroxide-conjugated anti-rabbit, anti-mouse, or anti-goat IgG antibodies (Cell Signaling, Danvers, MA, USA) for 1 hour. The blot was developed using Super-Signal Reagent (Pierce) and visualized with a Kodak Image Station 4000R. Blot densities were analyzed utilizing Kodak 1D software.

**Determination of CTRP/AdipoR1 binding**

CTRP9/AdipoR1 binding was determined as recently described by Denzel et al.$^7$ In brief, HUVECs were incubated with CTRP9 and cross-linked with 3.0 mmol/l dimethyl 3,3'-dithiopropionimidate dihydrochloride (DTBP, Pierce Biotechnology) for 30 minutes at room temperature. Cells were then washed and lysed (with lysis buffer containing 20 mmol/l Tris HCl pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l sodium pyrophosphate, 10 mmol/l beta glycerophosphate, 50 mmol/l sodium fluoride, protease inhibitor cocktail [Sigma-Aldrich], 0.1 mmol/l PMSF, and 1% NP40), and incubated with rabbit anti-CTRP9 antibodies at 4°C for 2 hour2. After addition of Magnetic Protein A beads (Invitrogen), samples were incubated at 4°C overnight. Beads were then collected
and washed 5 times in lysis buffer. Bound protein complexes were analyzed by Western blot.

**Tube Formation Assay**

Twenty-four-well culture plates were coated with growth factor-reduced Matrigel (BD Biosciences) per manufacturer’s instructions. HUVECs were seeded on coated plates at 5×10⁴ cells/well in medium containing gCTRP9 (3 µg/ml), VEGF (10 ng/ml), or BSA (10 µg/ml) and incubated at 37°C for 6 hours. Tube formation was observed and imaged. Tube formation degree was quantified by measuring tube length from each well via Image-Pro Plus 5.0 (BD Bioscience) in three randomly chosen fields.

**Effect of full length CTRP9 on endothelial dysfunction in diabetic animals:**

Adult (6-week old) male C57BL/6J mice were randomized to receive a high-fat diet (HFD, Research Diets Inc. D12492i, 60 kcal%) or a control normal diet (ND, D12450Bi, 10 kcal% fat) containing identical protein content as the high-fat diet. After 3 weeks, mice were treated with fCTRP9 or vehicle via Alzet mini-osmotic pump (1µg/g/d, Durect Corporation, Cupertino, CA) for another week. Endothelial function was determined by Ach-induced vasorelaxation.


I. Both globular domain of CTRP9 (gCTRP9) and full length CTRP9 (fCTRP9) enhance NOx production, AMPK phosphorylation, and eNOS phosphorylation in mouse aortic endothelial cells. mAEC incubation with globular domain of CTRP9 (0.3, 1, 3 µg/ml) for 15 minutes enhanced NO production (A) and AMPK phosphorylation (B) in concentration-dependent manner. Full length CTRP9 (fCTRP9), produced in human mammalian cells, enhanced NO production (C) and eNOS phosphorylation (D) in concentration-dependent manner.
II. (A) In dose- and AMPK-dependent fashion, gCTRP9 induces vasorelaxation in aortic vascular rings, an effect that is nullified in the presence of Compound C, an AMPK inhibitor. (B) CTRP9 plasma levels, assessed by western blot, in control and animals fed high fat diet (top segment). First bar (left) indicates control animal CTRP9 plasma levels. After 3 weeks of high fat diet, CTRP9 levels fell to approximately 40% of normal (second bar middle)). Supplementing exogenous fCTRP9 to the animals (concentration 1 µg/g body weight/day) for one week, levels of CTRP9 were restored to normal levels (third bar right).
III. Proposed signaling pathway and involved molecular mechanisms of CTRP9-induced vasorelaxation in aortic vascular rings and endothelial cells.