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

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Abstract

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 HUVECs. Of eight 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 upon CTRP9-induced AMPK phosphorylation, but blocked eNOS phosphorylation and NO production. Adiponectin receptor 1 (AdipoR1), but not receptor 2, knockdown blocked CTRP9-induced AMPK/Akt/eNOS phosphorylation and NO production. Finally, pre-incubating vascular rings with an AMPK-inhibitor abolished CTRP9-induced vasorelaxive effects.

Conclusion—We have provided the first evidence that CTRP9 is a novel vasorelaxive adipocytokine which may exert vasculoprotective effects via the AdipoR1/AMPK/eNOS dependent/NO mediated signaling pathway.

Keywords

Endothelial Function; Nitric Oxide; Diabetes; Signal Transduction

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The first two authors contributed equally to this study.

Disclosure:
None

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Introduction

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, while 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, an insulin-sensitizing adipokine predominantly secreted by adipocytes, possesses potent protective effects against endothelial dysfunction.[3] The positive association between serum adiponectin level and intact endothelium-dependent vasorelaxation has been demonstrated repeatedly.[4,5,6] Moreover, reduced plasma APN in diabetic patients has been demonstrated to be associated with endothelial dysfunction. Adiponectin knockout mice have been generated and studied by many groups. When metabolically challenged (e.g., high-fat diet), adiponectin-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.[7,8,9] These results suggest that potent compensatory mechanisms are in place.

Recently, a highly conserved family of adiponectin paralogs, designated C1q tumor necrosis factor (TNF) related proteins (CTRPs), has been discovered. Most of the known members (CTRPs 1 through 10) consist of four 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 adiponectin belong to the C1q/TNF protein superfamily, which continues to grow as more C1Q domain proteins are discovered.[12] Investigated for its structural similarity to adiponectin, the CTRP family has been demonstrated to have substantial metabolic effects similar to APN, particularly CTRP1 and 3.[12,13] Accordingly, it has recently been proposed that CTRPs, particularly CTRP1 and 3, may have partially overlapping metabolic function effectively compensating for adiponectin-deficient disease states.[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 vasorelaxive effects; 2) to compare the vasorelaxive 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 online supplementation.

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.

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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 agent) induced a concentration-dependent vasorelaxation similar to that elicited by acidified $NaNO_2$ (an endothelium-independent vasodilator). At the maximal concentration studied (100 μM), ACh and $NaNO_2$ 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 vasorelaxive response to ACh stimulation, while maintaining a normal dilatory response to acidified $NaNO_2$ (data not shown).

Administration of CTRP 1, 2, 4, 6, or 7 caused neither significant vasoconstriction nor vasorelaxation (data not shown) and were not further investigated. However, administration of CTRP 3, 5, or 9 caused significant vasorelaxation in a concentration-dependent fashion. Of the three vasoactive CTRPs, CTRP3 and CTRP5 exhibited vasorelaxive effect tantamount to APN (Figure 1B). CTRP9 demonstrated greatest vasorelaxive potency, exceeding that of APN. Specifically, CTRP9 (3 μg/ml) administration elicited significant vasorelaxation comparable to that caused by 10μg/ml APN. At the maximal concentration observed (i.e., 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 upon this observed potency, CTRP9 was utilized 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 vasorelaxive molecule production by endothelial cells, the aortic vascular endothelial layer was gently denuded as previously described.[16] As summarized in Figure 1C, CTRP9 administration failed to cause vasorelaxation, indicating that CTRP9 acts as an endothelium-dependent vasorelaxive agent. Finally, we determined whether NO is the endothelium-generated molecule that is responsible for mediating the vasorelaxive effects of CTRP9. Aortic vascular rings were pre-treated with L-NAME (100 μmol/L). As summarized in Figure 1D, pre-treatment with L-NAME completely blocked vascular vasorelaxive response to CTRP9.

CTRP9-stimulated NOx production and caused AMPK, Akt and eNOS phosphorylation in ECs

Having demonstrated that CTRP9 induces vasorelaxive 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; adiponectin has been shown to activate eNOS via AMPK signaling.[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 α 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 μg/ml, substantially increased NO production was observed within 15 minutes of CTRP9 administration, followed by modest increases tapering with time. Treatment with L-NAME (an eNOS inhibitor) abolished CTRP-induced NO production enhancement. The

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experiments were also conducted utilizing globular APN; CTRP9 exhibited nearly tripled potency compared to APN in eliciting NO production (Figure 2A). Moreover, CTRP9 administration had no effect upon expression level of AMPK, Akt, and eNOS. However, CTRP9 enhanced the phosphorylation of AMPK, Akt, and eNOS in dose dependent manner (Figures 2C, D, and E). Similar results were obtained in mouse aortic endothelial cells. mAEC incubation with CTRP9 (0.3, 1, 3 μg/ml) for 15 minutes enhanced NO production (online Figure 1A), AMPK phosphorylation (online Figure 1B), and eNOS phosphorylation (data not shown) in concentration-dependent manner. Additionally, full length CTRP9, produced in human mammalian cells, similarly enhanced NO production (online Figure 1C), eNOS phosphorylation (online Figure 1D), 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 online Figure 1 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. 48 hours after transfection with siRNA specifically targeting AMPK, HUVECs were incubated with CTRP9 (3 μg/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,C) 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] Since CTRP9 caused significant phosphorylation of both AMPK and Akt, we further determined the role of Akt in CTRP9-eNOS signaling. 48 hours after transfection with siRNA specifically targeting Akt, HUVECs were incubated with CTRP9 (3 μg/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.

CTRP9 activates AMPK in an-AdipoR1 dependent fashion

Two adiponectin receptors, AdipoR1 and AdipoR2, have been cloned,[19] and several putative cell surface molecules, including T-cadherin and calreticulin (CRT)/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 utilized siRNA to specifically downregulate endogenous AdipoR1 and AdipoR2 production. 48 hours after transfection with siRNA specifically targeting AdipoR1 and AdipoR2, HUVECs were incubated with either vehicle or CTRP9 (3 μg/ml) for 15 minutes. Western blot analysis confirmed siRNA-induced suppression of AdipoR1 and AdipoR2 by 60–75% (Figure 5A). As illustrated in Figure 5B, downregulation of AdipoR1, but not AdipoR2, significantly reduced CTRP9-induced phosphorylation of

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AMPK, Akt, and eNOS, as well as NO production (Figure 5C) in HUVECs. Moreover, no significant difference in these outcome parameters was observed between AdipoR2 knockdown, and AdipoR1 and AdipoR2 double-knockdown cells.

We next determined whether there was direct interaction between AdiopR1 and CTRP9. We incubated HUVECs with CTRP9 and cross-linked with DTBP, and used specific CTRP9 antibody to immunoprecipitate this protein alone with its binding partner. Indeed, AdiopR1 was co-immunoprecipitated in a complex with CTRP9 (Figure 5D), suggestive of a direct interaction involving CTRP9 binding to AdiopR1.

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 upon 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 $NaNO_2$ remained unchanged in these vessels (Figure 6C). These results indicate that high-fat diet-induced hyperlipidemia caused significant endothelial dysfunction. Moreover, as Supplemental Online Figure 2B 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, online Figure 2B) for one 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 upon CTRP9-induced vasorelaxive response. As summarized in online Figure 2A, pre-treatment with Compound C, a selective AMPK inhibitor, completely blocked CTRP9-induced vasorelaxation.

DISCUSSION

While 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 CORS26/cartducin of mainly chondrocyte origin, stimulates proliferation of chondrogenic precursor cells by activating ERK1/2 (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 andHT144 cells.[26] Secreted from adipose tissue, CTRP9 reduces serum glucose in mice, and

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forms heterotrimers with adiponectin.[27] CTRPs 8 and 10 are human specific, and their biologic function has not been previously studied. 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 CTRPs 3, 5, and 9 exert vasorelaxive effects, with CTRP9 being the most potent (possessing nearly three times the vasorelaxive effect of APN). CTRP9-mediated vasorelaxation is dependent upon 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 vasorelaxive agent.

To dissect the underlying mechanisms responsible for CTRP9-induced vascular dilation, we utilized 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. Endothelial nitric-oxide synthase (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 Ser^{1177}.[31,32] Our results demonstrate that CTRP9 enhances Akt/eNOS phosphorylation and NO production in an AMPK-dependent fashion. Furthermore, the vasorelaxive effect of CTRP9 was abolished by Compond C, an AMPK inhibitor (online Figure 2A), 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 (Figures 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 utilized 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 Ser^{473} (Figure 3B), but Akt suppression had no effect upon CTRP9-induced AMPK phosphorylation at Thr^{172} (Figure 4B). CTRP9's downstream effects of eliciting increased eNOS activity and NO production resulted in accelerated capillary-like tube formation (Figure 6A). Furthermore, utilizing an established high-fat-diet induced model of endothelial dysfunction, we demonstrated in vivo CTRP9 treatment for one 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 seven 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.[35,36,37] Aortic endothelial cells express both receptor types, but predominantly AdipoR1.[38] Previous to this study, whether CTRP9 interacts with one, both, or neither adiponectin receptor subtype was completely unknown. siRNA

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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,[36, 39,40] consistent with our study's mechanistic data. The adapter protein APPL1, containing a pleckstrin homology domain, a phosphotyrosine-binding domain, and a leucine zipper motif, appears to be a key intracellular effector of adiponectin's effects, via binding to the N-terminus of AdipoR1.[41,42] 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 vasorelaxive effects. While CTRPs 3 and 5 share vasorelaxive potency tantamount to APN, the vasorelaxive effect of CTRP 9 exceeds APN by nearly three-fold. CTRP9 elicits vasorelaxation in NO-dependent fashion. Finally, CTRP9 induces endothelial NO production in an AMPK-dependent manner, and AdipoR1 acts as a likely receptor of CTRP9 (summary online Figure 2B). 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 adipocytokine 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 physiologic 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 vasorelaxive agent that exceeding 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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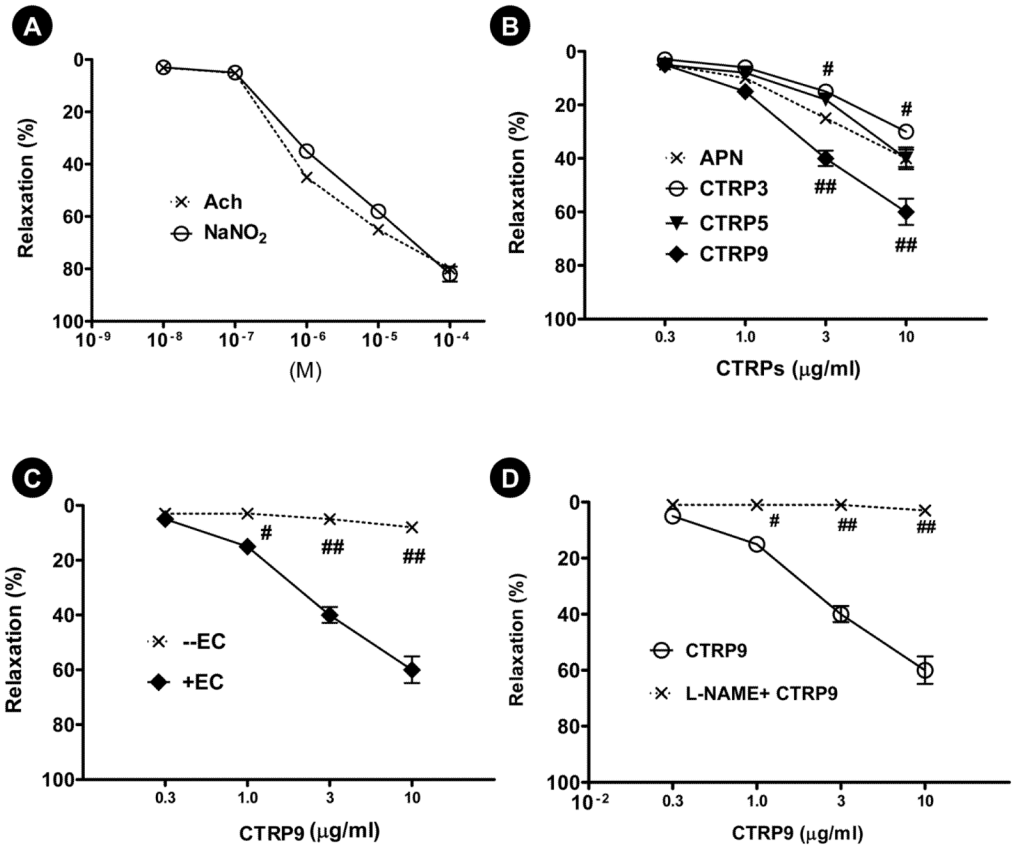

Figure 1. CTRPs induced vasorelaxation in aortic vascular rings

(A) ACh and $NaNO_2$ induced similar vasorelaxation in vascular segments with intact endothelial function. (B) Vasorelaxation in cumulative concentrations of CTRP3, 5, 9 and APN. gCTRP9 exhibited most potent vasorelaxive action; gCTRP3 and 5 have comparable vasorelaxive potency as APN. n=5–7 mice/group. #indicates P<0.05, ##indicates P<0.01. (C) gCTRP9 induced aortic vascular ring vasorelaxation with intact endothelium, but failed to do so in vascular rings without endothelium, evidencing CTRP9's vasorelaxive effects to be endothelium-dependent. (D) gCTRP9-induced aortic vascular ring vasorelaxation is blocked when rings were pre-treated with L-NAME (NO synthesis inhibitor, 100 μmol/L). n=5–7 mice/group. #indicates P<0.05, ##indicates P<0.01.

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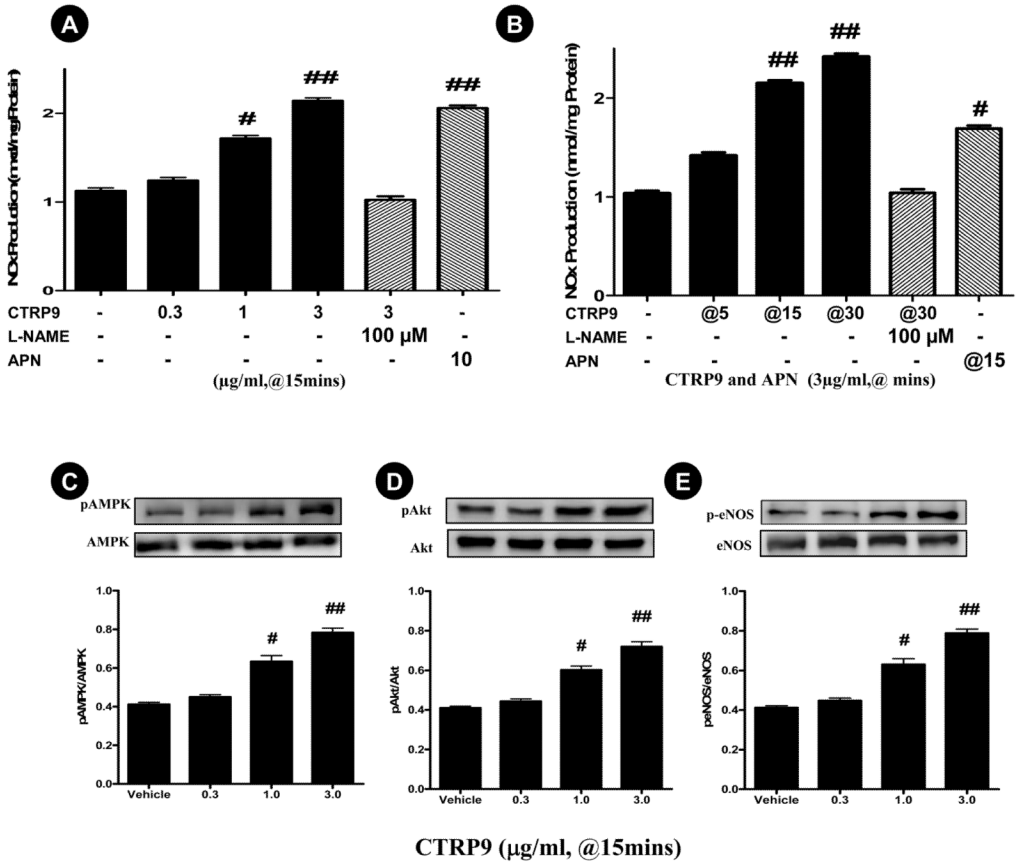

Figure 2. CTRP9 stimulated NO production, AMPK phosphorylation, and eNOS phosphorylation in time and dose dependent manner

(A) HUVEC incubation with gCTRP9 (0.3, 1, 3 μg/ml) or gAPN (10 μg/ml) for 15 minutes enhanced NO production in concentration-dependent manner. Treatment with L-NAME abolished gCTRP9-induced NO production enhancement. (B) Treatment of HUVECs with gCTRP9 or gAPN (3 μg/ml) for different time periods (5, 15, and 30 minutes) enhanced NO production, significantly increasing after 15 minutes, with increasing trend after 30 total minutes. Treatment with L-NAME abolished gCTRP9-induced NO production enhancement. HUVEC incubation with gCTRP9 (0.3, 1, 3 μg/ml) for 15 minutes enhanced AMPK (C), eNOS (D), and Akt (E) phosphorylation respectively. n=5–6 repeated experiments/condition. #indicates P<0.05, ##indicates P<0.01.

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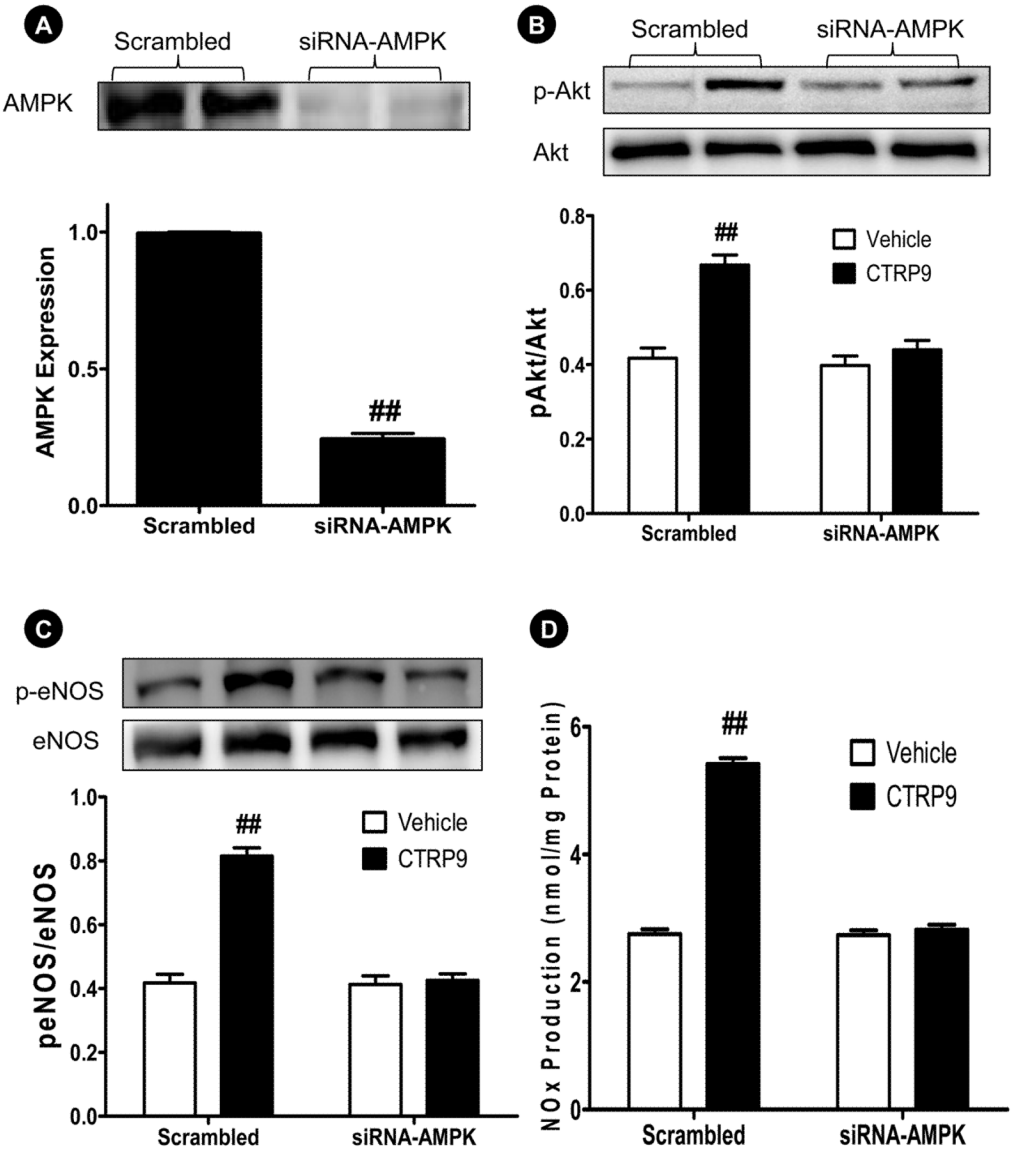

Figure 3. CTRP9-induced eNOS activation and NO production in HUVECs is AMPK dependent

After AMPK siRNA knockdown, HUVECs were incubated with gCTRP9 (3 μg/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=5–6 repeated experiments/condition. ##indicates P<0.01.

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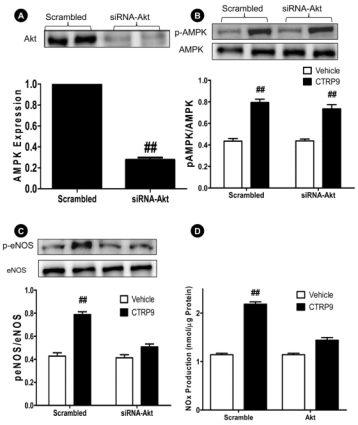

Figure 4. The role of Akt in CTRP9-induced eNOS activation and NO production in HUVECs

After Akt siRNA knockdown, HUVECs were incubated with gCTRP9 (3μg/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=5-6 repeated experiments/condition. ## indicates $P<0.01$.

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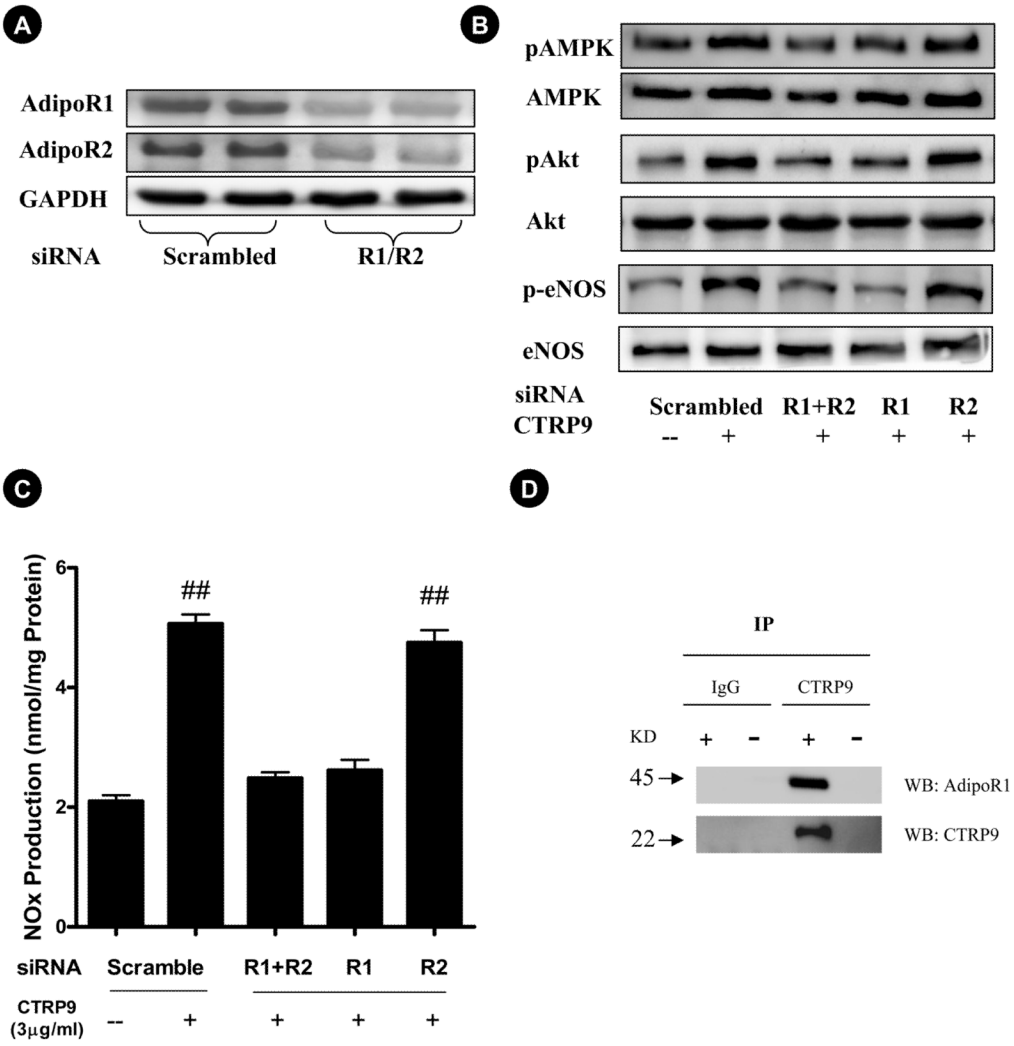

Figure 5. The role of AdipoR1 and R2 in CTRP9-induced eNOS activation and NO production in HUVECs

After Adipo R1 and R2 siRNA knockdown, HUVECs were incubated with gCTRP9 (3μg/ml) for 15 minutes. Western blot analysis confirmed siRNA induced significant AdipoR1 and R2 protein concentration reduction (A). Knockdown of both AdipoR1 and R2 or AdipoR1 alone reduced gCTRP9-induced AMPK/Akt/eNOS phosphorylation (B) and NO production (C), while down-regulation of AdipoR2 alone did not have significant effects. (D): Representative co-immunoprecipitation results showing that CTRP9 binds AdipoR1. HUVECs were incubated with (right lane, +) or without (−, left lane) CTRP9 and cross-linked with 3.0 mmol/l dimethyl 3,3′-dithiopropionimidate dihydrochloride (DTBP) for 30 minutes at room temperature. Samples were immunoprecipitated with CTRP9-specific antibody followed by immunoblotting with antibodies against AdipoR1 (top) or CTRP9 (bottom). The experiments were repeated 5–6 times/condition.

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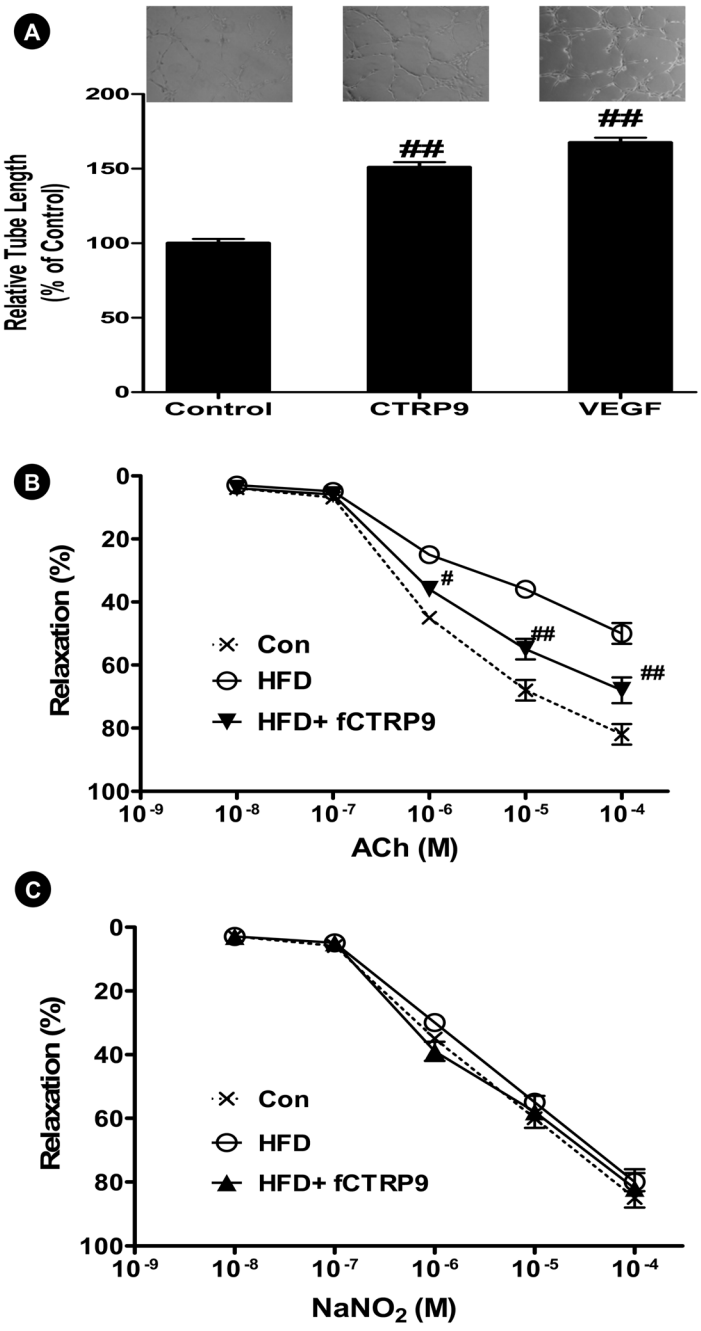

Figure 6. CTRP9 promoted endothelial cell tube formation in vitro, and improved endothelium-dependent vasorelaxation in high fat diet (HFD) fed mice when administered in vivo

(A): CTRP9 promoted endothelial cell differentiation into tube-like structures when HUVECs were plated upon Matrigel matrix. ##indicates P<0.01. In aortic segments from HFD (high fat diet) mice treated with vehicle, concentration-dependent vasorelaxation in response to ACh (B) is significantly attenuated whereas response to acidified $NaNO_2$ is normal (C), indicating that HFD-induced significant endothelial dysfunction. Treatment of HFD mice with fCTRP9 for 1 week significantly improved endothelial function as evidenced by downward-shift of concentration-dependent curve in response to ACh (B). n=5–7 mice/group. #P<0.05 and ## P<0.01 vs. HFD mice treated with vehicle.