Original Article

Adiponectin-Induced Endothelial Nitric Oxide Synthase Activation and Nitric Oxide Production Are Mediated by APPL1 in Endothelial Cells

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Adiponectin protects the vascular system partly through stimulation of endothelial nitric oxide (NO) production and endothelium-dependent vasodilation. The current study investigated the role of two recently identified adiponectin receptors, AdipoR1 and -R2, and their downstream effectors in mediating the endothelium actions of adiponectin. In human umbilical vein endothelial cells, adiponectin-induced phosphorylation of endothelial NO synthase (eNOS) at Ser^{1177} and NO production were abrogated when expression of AdipoR1 and -R2 were simultaneously suppressed. Proteomic analysis demonstrated that the cytoplasmic tails of both AdipoR1 and -R2 interacted with APPL1, an adaptor protein that contains a PH (pleckstrin homology) domain, a PTB (phosphotyrosine-binding) domain, and a Leucine zipper motif. Suppression of APPL1 expression by RNA interference significantly attenuated adiponectin-induced phosphorylation of AMP-activated protein kinase (AMPK) at Thr^{172} and eNOS at Ser^{1177}, and the complex formation between eNOS and heat shock protein 90, resulting in a marked reduction of NO production. Adenovirus-mediated overexpression of a constitutively active version of AMPK reversed these changes. In *db/db* diabetic mice, both APPL1 expression and adiponectin-induced vasodilation were significantly decreased compared with their lean littermates. Taken together, these results suggest that APPL1 acts as a common downstream effector of AdipoR1 and -R2, mediating adiponectin-evoked endothelial NO production and endothelium-dependent vasodilation. *Diabetes* 56:1387–1394, 2007

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AMPK, AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase; GFP, green fluorescent protein; HSP, heat shock protein; HUVEC, human umbilical vein endothelial cell; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PI, phosphoinositide; RNAi, RNA interference.

Endothelial dysfunction, characterized by decreased production and/or bioactivity of nitric oxide (NO) and impaired endothelium-dependent vasodilation, is a key mediator that links obesity, diabetes, and cardiovascular diseases (1). Dysfunction of the endothelium in conduit arteries is a well-established antecedent of hypertension and atherosclerosis, whereas dysfunction of peripheral vascular endothelium at the arteriolar and capillary level contributes to the pathogenesis of insulin resistance and the metabolic syndrome (2). On the other hand, insulin resistance aggravates endothelial dysfunction. Therapeutic interventions in animal models and humans have demonstrated that improving endothelial function ameliorates insulin resistance, while increasing insulin sensitivity alleviates endothelial dysfunction (3).

Adiponectin, an insulin-sensitizing adipokine secreted predominantly from adipocytes, possesses potent protective effects against endothelial dysfunction (4). Unlike most adipokines, plasma levels of adiponectin are decreased in obese individuals and patients with insulin resistance, type 2 diabetes, and cardiovascular diseases. An independent association between serum levels of adiponectin and endothelium-dependent vasodilation has been repeatedly documented (5–7). Hypoadiponectinemia has been closely linked to impairment in endothelium-dependent vasodilation in both normal subjects and patients with hypertension and type 2 diabetes. Consistent with these clinical findings, adiponectin-deficient mice exhibit reduced endothelium-dependent vasodilation on an atherogenic diet (6), increased neointimal hyperplasia after acute vascular injury (8,9), and elevated blood pressure compared with their wild-type littermates (10). On the other hand, both adenovirus-mediated overexpression of full-length adiponectin and transgenic overexpression of globular adiponectin result in a marked alleviation of atherosclerotic lesion in apolipoprotein E–deficient mice (11) and also cause a significant amelioration of endothelial dysfunction and hypertension (10) in obese mice.

The endothelium-protective functions of adiponectin are mediated, at least in part, by its ability to increase the production of NO, a vasodilator synthesized by endothelial NO synthase (eNOS) from the precursor L-arginine (4, 7,12). NO protects the vascular system by enhancing vasodilation and inhibiting platelet aggregation, monocyte adhesion, and smooth muscle cell proliferation (13). Recent studies from several independent laboratories have

demonstrated that adiponectin stimulates endothelial NO production and augments endothelium-dependent vasodilation (7,12,14–16). In endothelial cells, adiponectin enhances eNOS activity by inducing eNOS phosphorylation at Ser^{1177} and the complex formation between eNOS and heat shock protein (HSP) 90, through activation of AMP-activated protein kinase (AMPK) (12,15,16).

Two putative adiponectin receptors, AdipoR1 and -R2, have recently been cloned (17). These two receptors contain seven transmembrane domains, but they are structurally and functionally distinct from classical G-protein–coupled receptors. Both AdipoR1 and -R2 have an inverted membrane topology with a cytoplasmic NH_2 terminus and a short extracellular COOH terminus of ~25 amino acids (18). We have recently demonstrated that both AdipoR1 and -R2 are expressed in endothelial cells (7). Nevertheless, whether the endothelial actions of adiponectin are mediated by these two receptors remain to be determined.

In this study, we investigated the role of AdipoR1 and -R2 in the adiponectin-elicited signaling pathway that leads to increased NO production in human umbilical vein endothelial cells (HUVECs). Our results demonstrated that simultaneous downregulation of both receptors resulted in a marked attenuation of adiponectin-induced eNOS activation and NO production. Furthermore, we found that APPL1, an intracellular adaptor protein that contains an NH_2-terminal BAR (Bin/Amphiphysin/Rvs) domain, a PH (pleckstrin homology) domain, a COOH-terminal PTB (phosphotyrosine-binding) domain, and a Leucine zipper motif (19), acts as a signaling adaptor mediating adiponectin-evoked NO production by interacting with the cytoplasmic tails of AdipoR1 and -R2 in endothelial cells.

RESEARCH DESIGN AND METHODS

Anti–phospho-AMPKα (Thr^{172}) and anti–total AMPKα antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti–green fluorescent protein (GFP) and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, California). pEGFP-C3 vector, anti–phospho-eNOS (Ser^{1177}), anti-eNOS antibodies, and ECGS (endothelial cell growth supplement) were obtained from BD Transduction Laboratories (San Jose, CA). Anti-FLAG M2 antibody was from Sigma Aldrich (St. Louis, MO). Recombinant murine globular and full-length adiponectin was produced from HEK293 cells and *Escherichia coli* as we previously described (20–22). The endotoxin was removed by a Detoxi-gel endotoxin-removal kit (Pierce). Anti-AdipoR1 and anti-AdipoR2 antibodies were from Abcam (Cambridge, MA) and Alpha Diagnostics (San Antonio, CA), respectively. Anti–human APPL1 antibody was produced by immunization of New Zealand female rabbits with the recombinant full-length human APPL1 produced from *E. coli*, using the protocol as we previously described (20). The antibody was affinity-purified with Sepharose 4B beads coupled with recombinant human APPL1.

Cell culture, transfection, and adenoviral infection. HUVECs at passages 4–8 were cultured on gelatin-coated flasks in M199 supplemented with 15% fetal bovine serum, 0.1 mg/ml heparin, and 0.03 mg/ml endothelial cell growth supplement. Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% new calf serum. For plasmid transfection, HUVECs (3×10^6 cells) were trypsinized and resuspended in 400 μl M199 with 20 μg plasmid DNA on ice for 10 min, and they were then transferred into a 0.4-cm electroporation cuvette and electroporated at 1,000 μF and 200 V using a Bio-Rad Gene Pulser instrument. After electroporation, the cells were incubated at room temperature for 10 min and seeded into the culture dishes.

Recombinant adenoviruses for expression of a constitutively active or dominant-negative version of AMPKα were kindly provided by Dr. D. Carling (23). Constitutively active AMPKα is the truncated form of AMPK catalytic subunit α1 with threonine 172 being replaced by aspartic acid, whereas dominant-negative AMPKα carries a single mutation (aspartate 157 replaced by alanine). Recombinant adenovirus encoding luciferase was described previously (24). HUVECs were infected with these adenoviruses at 50 pfu/cell.

Stealth RNA preparation and transfection. Duplex stealth RNA interference (RNAi) for AdipoR1, AdipoR2, APPL1, and scrambled RNAi were purchased from Invitrogen and are listed in supplemental Table 1, which can be found in an online appendix (available at http://dx.doi.org/10.2337/db06-1580). These oligonucleotides were transfected into HUVECs using oligofectamine according to the manufacturer's instructions (Invitrogen). Total RNA was extracted from cells using an RNA extraction kit (Viogene) and was reverse-transcribed using an ImProm-II reverse transcription system (Promega, Madison, WI). The relative mRNA abundance of AdipoR1, AdipoR2, APPL1, and human glyceraldehyde-3-phosphate dehydrogenase were analyzed by real-time quantitative PCR using a fluorescent *Taq*Man 5′-nuclease assay on an Applied Biosystems Prism 7000 sequence detection system.

Construction of expression vectors. cDNA encoding the full-length and truncated versions of human APPL1 and AdipoR1 and -R2 were cloned from the HUVEC cDNA library using the primers listed in supplemental Table 2. The amplified cDNA fragments were subcloned into pcDNA 3.1^+ or pEGFP-C3 to generate various vectors for mammalian expression of FLAG- or GFP-tagged proteins. The cDNA fragments encoding the NH_2-termini of human AdipoR1 (from amino acids 1–134) and AdipoR2 (from amino acids 1–145) were also subcloned into pGEX4T-1 to produce vectors GST-AdipoR1-C and GST-AdipoR2-C for prokaryotic expression of these two proteins with GST tagged at their NH_2-termini, respectively. These two GST-tagged fusion proteins were expressed in BL21 bacterial cells and affinity-purified with glutathione Sepharose 4B beads.

Coimmunoprecipitation and Western blot analysis. Cells transiently transfected with various expression vectors were solubilized in a lysis buffer containing 20 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 1 mmol/l Na_2EDTA, 1 mmol/l EGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, and the complete protease inhibitor cocktail. Cell lysates were clarified by centrifugation at 12,000 rpm at 4°C for 15 min and were then preincubated with 50 μl protein A/G beads at 4°C for 1 h to remove nonspecific bindings. The remaining supernatant was incubated with various antibodies at 4°C overnight, and the immunocomplexes were precipitated by adding 50 μl of protein A/G beads at 4°C for 2 h. The beads were washed with 1 ml lysis buffer four times, and the immunocomplexes bound to the beads were eluted by boiling in 100 μl SDS-PAGE loading buffer. The eluted samples were resolved by SDS-PAGE, transferred onto polyvinylidene fluoride membrane, and probed with different primary antibodies as indicated. The proteins were visualized using chemiluminescence detection.

GST fusion protein pull-down. HEK293 cells were transiently transfected with plasmids encoding the FLAG-tagged full-length NH_2 terminus or COOH terminus of APPL1. At 48 h after transfection, cells were solubilized in a lysis buffer, and cell lysates were clarified by centrifugation. Then, the 10-μg GST-tagged cytoplasmic tail of AdipoR1 or -R2 was immobilized onto glutathione Sepharose 4B beads by incubation at 4°C for 2 h. The beads were reacted with cell lysates at 4°C overnight. After extensive washing with the lysis buffer, the bounded proteins were eluted with 5 mmol/l reduced glutathione, separated by SDS-PAGE, and visualized by silver staining. The proteins selectively bound to the COOH termini of AdipoR1 and -R2 were subjected to tandem mass spectrometry analysis to identify the nature of the protein, as we recently described (25,26). Alternatively, proteins separated by SDS-PAGE were transferred onto a nylon membrane and probed with anti-FLAG monoclonal antibody.

Measurement of NO production. Monolayer cells were grown in six-well dishes until 90% confluence and then starved in a serum-free medium for 4 h. The cells were treated with various concentrations of adiponectin, and the supernatants were collected at different time intervals. NO release was determined by measurement of nitrite (NO_2^-) and nitrate (NO_3^-) levels using a Sievers NO analyzer (Boulder, CO). The cells were collected for determination of protein concentrations using a bicinchoninic acid protein assay method (Pierce). NO levels were calculated as picomoles of NO per minute per mg protein, and they were expressed as the fold over the control conditions.

Vessel preparation and isometric tension measurement. Male C57BL/KsJ *db/db* diabetic mice aged 20–22 weeks, and their lean littermates were used for this study. The mice were killed by an overdose of pentobarbitone (60 mg/kg i.p.). The superior mesenteric arteries were dissected under the microscope. Each artery was cut into several rings ~2 mm in length in cold Krebs-Ringer bicarbonate solution with the following composition (in mmol/l): 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 11.0 dextrose, and 1.8 $CaCl_2$, aerated with 95% O_2 and 5% CO_2. The rings were suspended between two tungsten wires in a Myograph System (Danish Myo Technology, Aarhus, Denmark) for the recording of changes in isometric tension as previously described (15,27). Each ring was stretched in a stepwise manner to an optimal tension that was determined in length-active tension relationship experiments. After equilibration for 60 min, the rings were then precontracted with a submaximal concentration of phenylephrine. After a stable contraction was achieved, the rings were exposed to cumulative concentrations of adiponectin to evaluate endothelium-dependent vasodilation. N^{ω}-nitro-L-arginine methyl

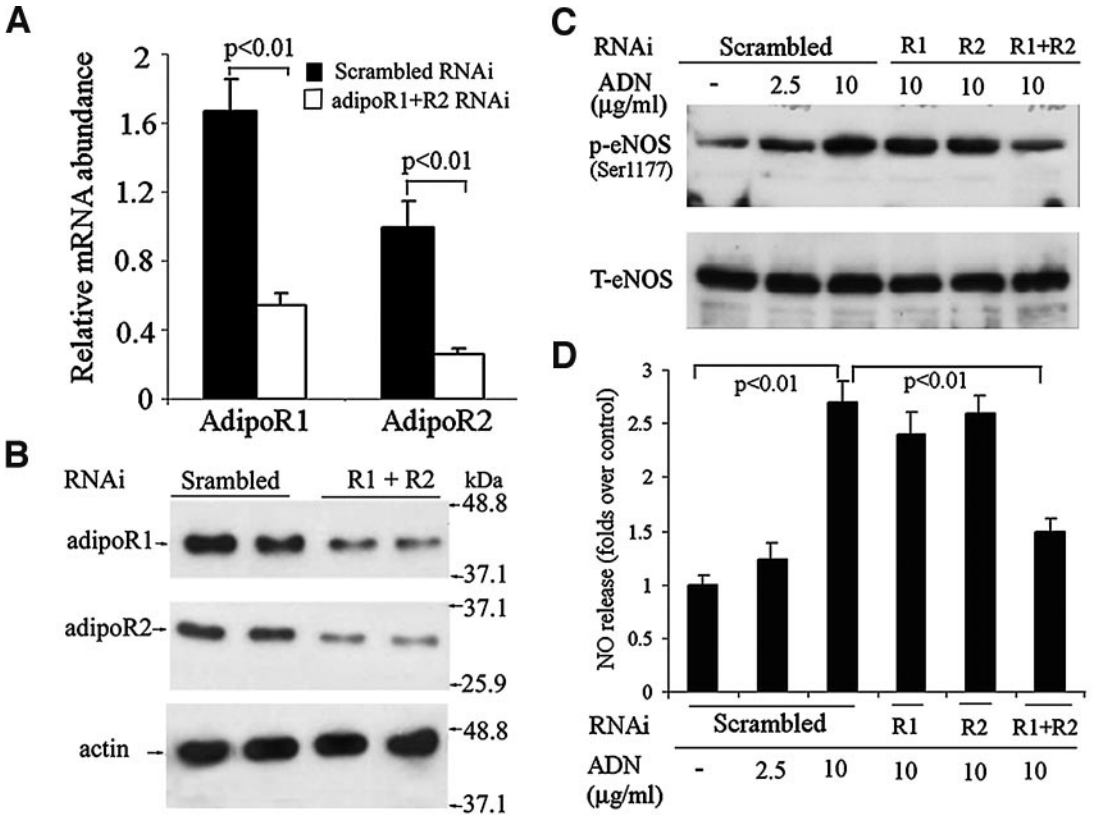

FIG. 1. Role of AdipoR1 and -R2 in adiponectin-induced eNOS phosphorylation at Ser^{1177} and NO production in HUVECs. Cells were transfected with target-specific RNAi duplexes for AdipoR1, AdipoR2, or scrambled RNAi as control. *A*: Real-time PCR to quantify the relative mRNA abundance of AdipoR1 and -R2 at 48 h after transfection. *B*: Western blot analysis to detect the protein levels of AdipoR1, AdipoR2, and actin (as control) in cells transfected with RNAi. *C*: Cells transfected with different RNAi duplexes were treated with adiponectin for 15 min, and 50 μg of proteins from cell lysates was separated by SDS-PAGE, transferred onto nylon membrane, and probed with anti–phospho-eNOS (Ser^{1177}) or anti–total eNOS antibody. *D*: NO release in the conditioned medium was measured at 60 min after adiponectin treatment. The data were expressed as the fold over the control cells treated without adiponectin (n = 5–6). AND, adiponectin; p-eNOS, phospho-eNOS; T-eNOS, total eNOS.

ester (L-NAME, 100 μmol/l), an inhibitor of NO synthesis, was used to confirm NO-mediated vasodilation.

Statistical analysis. The results are the means ± SD. Differences between groups were determined by Student's t test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Adiponectin-induced eNOS phosphorylation at Ser^{1177} and NO production are mediated through AdipoR1 and -R2 in endothelial cells. Our previous study demonstrated that both AdipoR1 and -R2 are expressed in human endothelial cells (7). Here, we investigated whether the endothelial actions of adiponectin are mediated by these two receptors. Quantitative real-time PCR analysis revealed that the mRNA abundance of AdipoR1 is 1.7-fold higher than that of AdipoR2 in HUVECs. The mRNA expression of AdipoR1 and -R2 was suppressed by 68 and 75%, respectively, at 48 h after transfection with the duplex stealth RNAi against both genes (Fig. 1*A*). Western blot analysis showed that the protein levels of AdipoR1 and -R2 were also decreased by RNAi specific to these two genes (Fig. 1*B*)

Consistent with previous findings (12,14–16), treatment with both full-length adiponectin (Fig. 1*C* and *D*) and globular adiponectin (supplementary Fig. 1) significantly induced eNOS phosphorylation at Ser^{1177} and NO production in HUVECs. The potency of full-length adiponectin produced from *E. coli* and mammalian cells is similar (data not shown), suggesting that the posttranslational modifications and the formation of high-order oligomeric structure is not required for this action. Downregulation of either AdipoR1 or -R2 alone had no significant effects on adiponectin-induced eNOS phosphorylation at Ser^{1177} and NO production. On the other hand, the effect of adiponectin was markedly attenuated when expression of AdipoR1 and -R2 was simultaneously suppressed.

Both AdipoR1 and -R2 interact with APPL1 in HUVECs. To identify the proximal downstream effectors of these two receptors, we expressed GST-tagged cytoplasmic tails of AdipoR1 (GST-AdipoR1-C) and AdipoR2 (GST-AdipoR2-C) for pull-down purification of their potential interaction proteins in HUVECs. Tandem mass spectrometry–based analysis identified several candidate proteins that are potentially associated with the cytoplasmic tails of AdipoR1 and/or AdipoR2, including phosphatase 2A, rabenosyn-5, striatin, GRS (regulator of G-protein signaling 4), and APPL1. Among these candidates, APPL1, a ~80-kDa adaptor protein that has previously been shown to interact with several membrane receptors and signaling molecules (19,28–30), bound to both GST-AdipoR1-C and GST-AdipoR2-C, but not GST alone. During the course of this study, APPL1 was also reported to interact with AdipoR1 and -R2 in C2C12 myotubes in a yeast two-hybrid screening (31).

To confirm the interaction between AdipoR1/AdipoR2 and APPL1, we coexpressed FLAG-tagged AdipoR1 or -R2

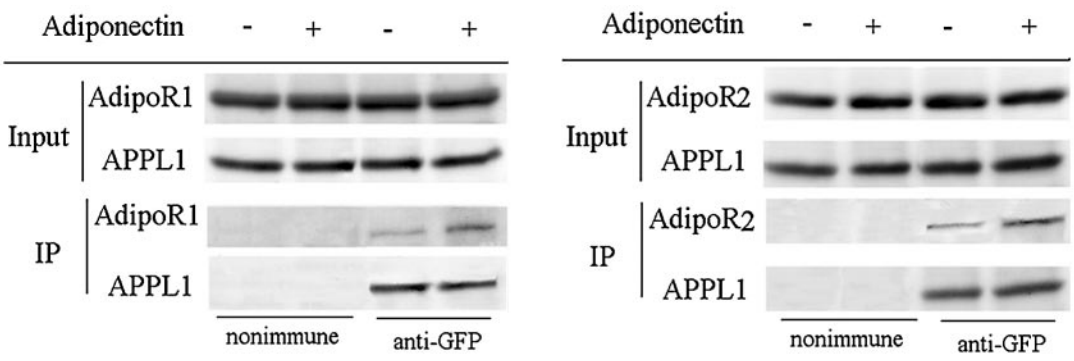

FIG. 2. Both AdipoR1 and -R2 are physically associated with APPL1 in HUVECs. Cells were cotransfected with vectors for GFP-tagged APPL1 and FLAG-tagged AdipoR1 (*A*) or AdipoR2 (*B*). At 42 h after transfection, cells were serum-starved for 6 h and then treated with adiponectin (10 μg/ml) for 15 min. Cell lysates were subjected to immunoprecipitation (IP) using anti-GFP monoclonal antibody or nonimmune mouse IgG as control, and the proteins bound to the antibodies were eluted, separated by SDS-PAGE, and detected with either anti-FLAG or anti-GFP antibody.

and GFP-tagged APPL1 in HUVECs. Coimmunoprecipitation experiments showed that both AdipoR1 and -R2 were physically associated with APPL1 (Fig. 2). Adiponectin moderately increased the interactions between APPL1 and both adiponectin receptors. Furthermore, the interaction between APPL1 and the two receptors was reproducibly observed when FLAG-tagged AdipoR1 and -R2 was immunoprecipitated with an anti-FLAG monoclonal antibody (data not shown).

Further analysis revealed that AdipoR1 and -R2 were bound by the COOH fragment that contains a PTB domain, but not by the NH_2-terminal region, which includes the BAR and PH domains (Fig. 3). Taken together, these results suggest an interaction between the cytoplasmic tail of the two adiponectin receptors and the COOH terminus of APPL1 in HUVECs.

Suppression of APPL1 expression by RNAi attenuates adiponectin-induced phosphorylation of AMPKα at Thr^{177} and of eNOS at Ser^{1177}, and NO production in HUVECs. Previous studies have shown that adiponectin stimulates NO production through activation of the AMPK/eNOS signaling cascade (12,16). We next investigated the role of APPL1 in this signaling pathway in HUVECs. At 48 h after transfection of cells with the duplex stealth RNAi against APPL1, its mRNA expression was decreased by ~76%. Western blot analysis confirmed that the protein concentration of APPL1 was also markedly reduced (Fig. 4*A*). Suppression of APPL1 expression resulted in a significant attenuation in adiponectin-evoked phosphorylation of AMPK at Thr^{172} and eNOS at Ser^{1177}, and production of NO (Fig. 4*B* and *C*).

Association of HSP90 with eNOS is an important step for maximal activation of eNOS activity (13). Adiponectin has been shown to enhance the complex formation between these two proteins in endothelial cells (15), although the underlying mechanisms remain to be determined. We next investigated the role of APPL1 in regulating the complex formation between eNOS and HSP90 in HUVECs. Coimmunoprecipitation analysis showed that treatment with adiponectin augmented the binding of HSP90 to eNOS (Fig. 5). On the other hand, adiponectin-evoked complex formation between HSP90 and eNOS was largely abrogated when expression of APPL1 was suppressed.

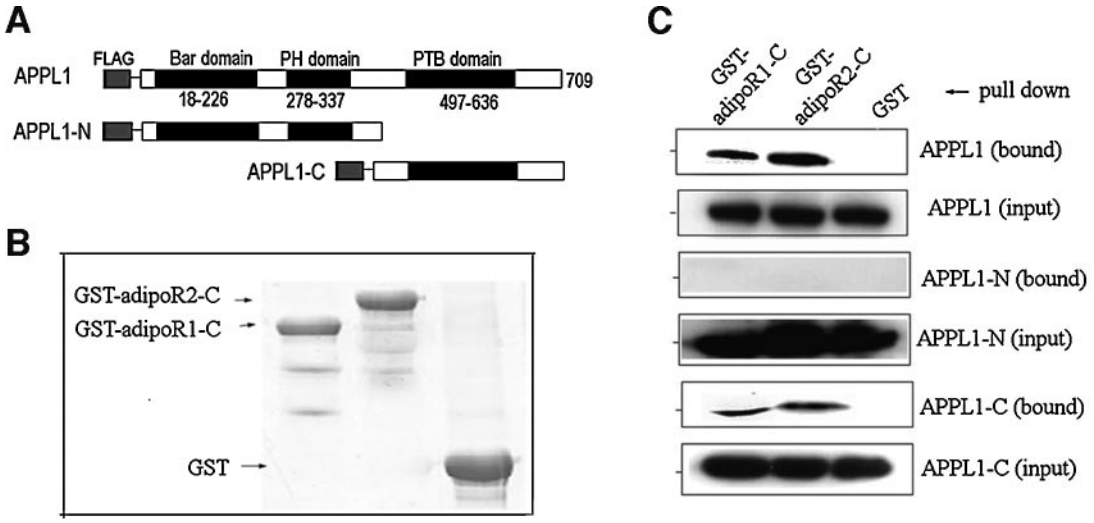

FIG. 3. The COOH domain, but not the NH_2-terminal region of APPL1, interacts with the cytoplasmic tails of AdipoR1 and -R2. *A*: Schematic presentation of the domain organization of wild-type and truncated APPL1 used in this study. *B*: SDS-PAGE analysis of GST and GST-tagged cytoplasmic tail of AdipoR1 (GST-AdipoR1-C) and AdipoR2 (GST-AdipoR2-C) expressed in *E. coli*. *C*: HEK293 cells were transfected with the expression vectors encoding FLAG-tagged full-length APPL1 or NH_2 terminus (APPL1-N) or COOH terminus of APPL1 (APPL1-C), as in panel *A*, for 48 h. Equal amount of cell lysates were incubated with glutathione Sepharose beads coupled for 2 h with 10 μg of GST or GST-tagged cytoplasmic tails of AdipoR1 or -R2. After extensive washing, proteins bound to the beads were eluted with reduced glutathione, separated by SDS-PAGE, and detected with an anti-FLAG monoclonal antibody.

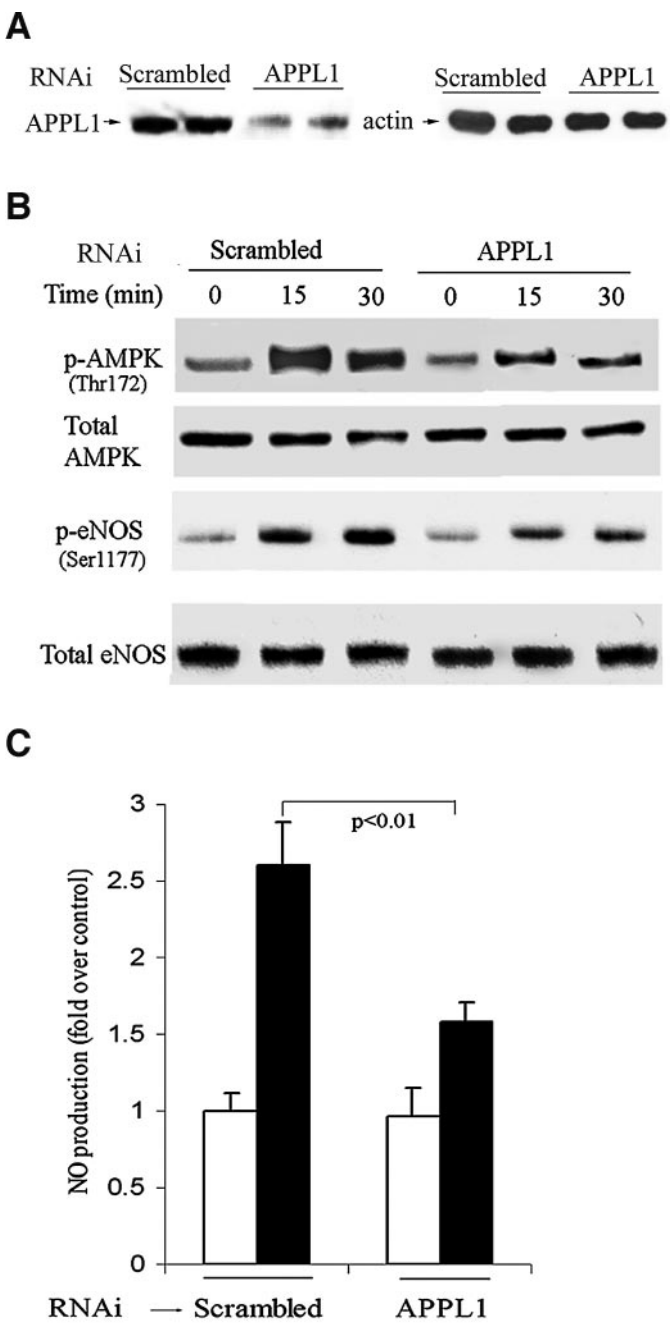

FIG. 4. APPL1 is required for adiponectin-evoked phosphorylation of AMPK at Thr^{172} and eNOS at Ser^{1177} as well as NO production in HUVEC cells. Cells were transfected with the target-specific RNAi duplexes for APPL1 or scrambled RNAi as control. *A*: Western blot analysis to detect the protein levels of APPL1 and actin at 48 h after transfection. *B*: At 42 h after transfection, cells were starved in a serum-free medium for 6 h and then stimulated with adiponectin (10 μg/ml) for different time periods as indicated. Then, 50 μg protein from cell lysates were separated by SDS-PAGE and probed with different antibodies as indicated. *C*: NO production at 60 min after stimulation with adiponectin was measured as in Fig. 1 ($n = 5$). p-eNOS, phospho-eNOS; p-AMPK, phospho-AMPK. □, control; ■, adiponectin.

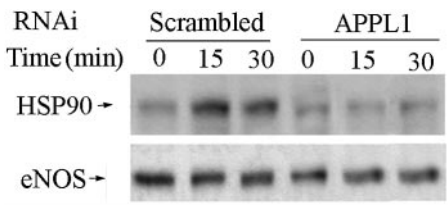

FIG. 5. Downregulation of APPL1 expression attenuates adiponectin-evoked association of HSP90 with eNOS in HUVECs. Cells were transfected with RNAi duplexes as in Fig. 4. The suppression efficiency of RNAi against APPL1 was confirmed by both real-time PCR and Western blot. At 42 h after transfection, cells were starved in serum-free medium for another 6 h, stimulated with adiponectin (10 μg/ml), and then dissolved in a lysis buffer as described in RESEARCH DESIGN AND METHODS. Cell lysates were subjected to immunoprecipitation using anti-eNOS antibody. The immunoprecipitated complexes were separated by SDS-PAGE and probed with the anti-HSP90 or -eNOS antibody as indicated.

Constitutive activation of AMPK is sufficient to mimic the effects of adiponectin in eNOS activation and NO production. It is now known that adiponectin exerts many biological actions through activation of AMPK, such as enhancing lipid β-oxidation and insulin sensitivity in skeletal muscle (32), inhibiting gluconeogenesis in liver (17), and protecting against acute cardiac injury (33). AMPK activation can stimulate eNOS activity by enhancing its association with HSP90 (34) as well as inducing its phosphorylation at Ser^{1177} (35,36). We next investigated the role of AMPK in adiponectin-induced eNOS activation and its relationship with APPL1 in HUVECs. Consistent with the two previous reports (12,16), adenovirus-mediated overexpression of a dominant-negative version of AMPK (23) completely blocked adiponectin-evoked association of eNOS with HSP90 as well as phosphorylation of eNOS at Ser^{1177} (Fig. 6). On the other hand, overexpression of a constitutively active version of AMPK alone was sufficient to mimic the effects of adiponectin to induce eNOS-HSP90 complex formation and eNOS phosphorylation at Ser^{1177}, even when APPL1 expression was suppressed. Our coimmunoprecipitation experiment showed that there was no direct interaction between APPL1 and HSP90 or eNOS. Taken together, these results suggest that APPL1 mediates adiponectin-induced association of eNOS and HSP90 via an indirect mechanism, possibly through activation of AMPK in endothelial cells.

APPL1 expression and adiponectin-evoked vasodilation are decreased in small mesenteric arteries of *db/db* diabetic mice. To explore the pathophysiological relevance of the above in vitro findings, we next compared APPL1 mRNA expression in several tissues in C57BL/KsJ *db*/+ lean mice and *db/db* diabetic mice, an established animal model with endothelial dysfunction (27,37). *db/db* diabetic mice showed significantly increased body weight (52.4 ± 3.3 vs. 29.1 ± 1.6 g, $P < 0.01$) and higher plasma levels of blood glucose (483.6 ± 24.7 vs. 197.2 ± 11.5 mg/dl, $P < 0.01$), triglycerides (238.9 ± 15.2 vs. 92.5 ± 6.8 mg/dl, $P < 0.01$), insulin (14.3 ± 1.3 vs. 1.2 ± 0.1 ng/ml, $P < 0.01$), and cholesterol (164.8 ± 9.2 vs. 81.7 ± 7.3 mg/dl, $P < 0.01$) compared with their lean littermates. The mRNA expression level of APPL1 in small mesenteric arteries of *db/db* diabetic mice was significantly decreased (Fig. 7*A*). On the other hand, the expression levels of APPL1 in liver, kidney, and adipose tissues were comparable between *db/db* diabetic mice and their lean littermates (data not shown).

In agreement with a previous report (15), our results also showed that adiponectin dose-dependently induced vasodilation of small mesenteric arteries (Fig. 7*B*). Adiponectin at the concentrations of 5 and 10 μg/ml elicited 27.7 and 42.6%, respectively, vasorelaxation of small mesenteric arteries derived from the lean mice. The relaxation effect of adiponectin was endothelium dependent and

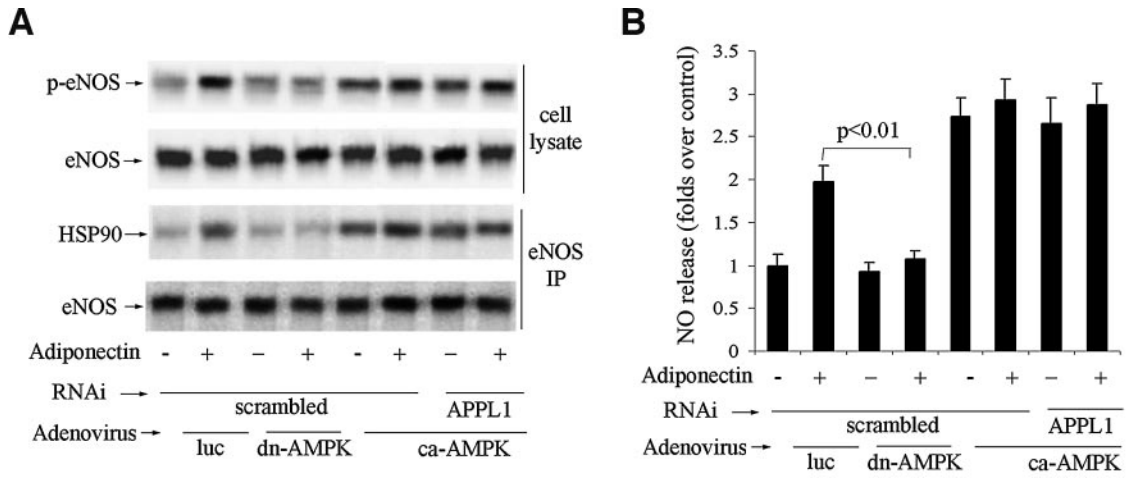

FIG. 6. Role of AMPK in adiponectin-induced eNOS phosphorylation at Ser^{1177}, association of eNOS with HSP90, and NO production in HUVECs. *A*: Cells were transfected with the RNAi duplexes as indicated and were then infected with recombinant adenovirus for expression of luciferase (luc), dominant-negative AMPK (dn-AMPK), and constitutively active AMPK (ca-AMPK) (50 pfu/cell). The suppression efficiency of siRNA against APPL1 was confirmed by both real-time PCR and Western blot. At 42 h after transfection, cells were starved in serum-free medium for 6 h and then treated with 10 μg/ml adiponectin for 15 min. Total cell lysates were separated by SDS-PAGE and probed with anti-eNOS and anti–phospho-eNOS antibody, or were subjected to immunoprecipitation using anti-eNOS antibody. The immunoprecipitated complexes were separated by SDS-PAGE and probed with anti-HSP90 or anti-eNOS antibody. *B*: NO released into conditioned medium was detected as in Fig. 1 (n = 4–6). p-eNOS, phospho-eNOS.

abolished by treatment with L-NAME (100 μmol/l), suggesting a primary role of NO in adiponectin-induced vasodilation. Notably, adiponectin produced significantly less relaxation in *db/db* diabetic mice than in their lean littermates. Taken together, these results suggest that decreased APPL1 expression in small mesenteric arteries may be closely related to impaired vasodilation in *db/db* diabetic mice.

DISCUSSION

In this study, we provided the first direct evidence showing that AdipoR1 and -R2, two putative adiponectin receptors, are involved in mediating adiponectin-evoked eNOS activation and NO production in endothelial cells. This conclusion is supported by our finding that adiponectin-induced eNOS phosphorylation at Ser^{1177} and NO production in HUVECs were abolished by simultaneous suppression of AdipoR1 and -R2 (Fig. 1). Suppression of each receptor alone had no obvious effect, suggesting that AdipoR1 and -R2 may compensate for each other's function in HUVECs. Alternatively, the incomplete suppression of these two receptors in our experiments might also account for the lack of obvious effect after suppression of each individual receptor. Because of the relatively low transfection efficiency in HUVECs, expression of AdipoR1 and -R2 was only suppressed by 68 and 75%, respectively, after transfection with RNAi against their respective gene. It is still possible that complete ablation of either AdipoR1 or -R2 individually is sufficient to blunt adiponectin-mediated eNOS phosphorylation and NO production. Further study on endothelial cells derived from AdipoR1 or -R2 knockout mice should help clarify the role of each receptor in adiponectin-induced NO production.

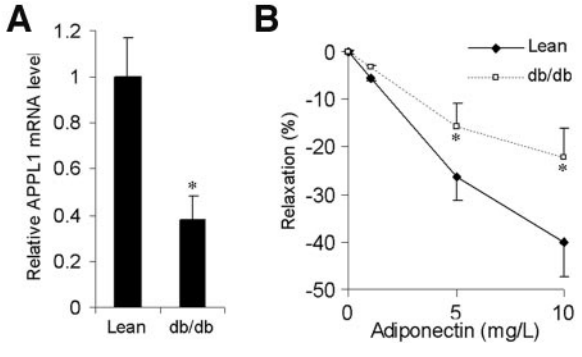

FIG. 7. APPL1 expression (*A*) and adiponectin-mediated vasodilation (*B*) in small mesenteric arteries are decreased in *db/db* diabetic mice. Small mesenteric arteries were isolated from male *db/db* obese/diabetic mice and their lean littermates. APPL1 mRNA expression in small mesenteric arteries was determined by real-time PCR and normalized against 18S RNA. Adiponectin-induced relaxations are shown as a percentage of the maximum contraction to phenylephrine. *P < 0.01 vs. lean controls (n = 5–6).

We identified APPL1 as an intermediate adaptor linking adiponectin receptors and the downstream signaling events that lead to increased NO production. APPL1 (also called DIP13α) was originally cloned in two-hybrid screens as an interacting partner of Akt2 (19) and the tumor suppressor DCC (deleted in colon cancer) (28). This protein contains multiple regulatory motifs that are involved in various signaling pathways. APPL1 is physically associated with several membrane receptors, such as follicle-stimulating hormone receptor (30) and androgen receptor (29), and the small GTPase Rab5 (38). In response to extracellular stimuli, such as epidermal growth factor and oxidative stress, APPL1 dissociates with Rab5 and translocates from membranes to the nucleus, where it interacts with nucleosome remodeling and histone deacetylase multiple protein complex NuRD/MeCP1, which in turn regulates chromatin structure and gene regulation (38). In addition, the interaction between APPL1 and the neurotrophin receptor TrkA is required for nerve growth factor–mediated signal transduction in neuronal cells (39). During the course of our study, Mao et al. (31) reported the interaction between the two adiponectin receptors and APPL1 in C2C12 myotubes in a yeast two-hybrid screen. In myotubes, APPL1 acts as a key mediator involved in adiponectin-evoked AMPK activation, lipid oxidation, membrane translocation of GLUT4, and glucose uptake (31). In agreement with this finding,

our proteomics-based analysis also demonstrated the interaction between the cytoplasmic tails of AdipoR1/AdipoR2 and APPL1 in endothelial cells. Furthermore, we showed that suppression of APPL1 expression by RNAi led to the abrogation of adiponectin-evoked eNOS activation and NO production, suggesting a critical role of this adaptor protein in mediating the vasodilating effects of adiponectin.

Growing evidence suggests that AMPK, a central regulator of cellular energy metabolism, plays a key role in modulating vascular reactivity. AMPK stimulates eNOS activity by phosphorylating eNOS at Ser^{1177} and promoting its association with HSP90. Several vasoprotective agents, such as the antidiabetic drug metformin (40), 17β-estradiol (34), high-density lipoprotein, and apolipoprotein AI (41), have recently been shown to activate AMPK in endothelial cells. Consistent with the previously reported findings (12,16), our results also demonstrated that AMPK is the principal kinase responsible for adiponectin-evoked eNOS phosphorylation at Ser^{1177}, eNOS association with HSP90, and NO production in HUVECs (Fig. 6). In addition, we found that APPL1 acts as an intermediate adaptor that links adiponectin receptors with AMPK activation. This notion is supported by our finding that adiponectin-induced AMPK phosphorylation at Thr^{172} was abrogated by RNAi-mediated downregulation of APPL1 expression, whereas overexpression of the constitutively active form of AMPK alone was sufficient to stimulate eNOS activation and NO production, even when APPL1 expression was suppressed (Fig. 5).

The precise mechanism by which the binding of APPL1 leads to the activation of AMPK remains to be defined. Recent studies have implicated the potential involvement of APPL1 in the regulation of phosphoinositide (PI) 3-kinase activity. APPL1 can directly interact with the regulatory subunit of PI 3-kinase (P85), and this interaction might be involved in androgen receptor–mediated activation of Akt (29). The selective PI 3-kinase inhibitors could abrogate adiponectin-evoked phosphorylation of AMPK at Thr^{172} and of eNOS at Ser^{1177}, and NO production in endothelial cells (12,15), suggesting that the activation of AMPK/eNOS by adiponectin is mediated by PI 3-kinase. A more recent study revealed that activation of the AMPK/eNOS pathway induced by the antidiabetic drug metformin is also dependent on PI 3-kinase (40). Whether PI 3-kinase is the downstream target of APPL1 is currently under investigation in our laboratory.

Endothelial dysfunction is a pathological condition closely associated with obesity and diabetes. Several factors, such as glucotoxicity, lipotoxicity, inflammation, and hypoadiponectinemia have been proposed to play an etiological role in obesity and diabetes-associated endothelial dysfunction (2,4). Nevertheless, the cellular mechanisms underlying this disorder remain poorly understood. In this study, we showed that adiponectin-mediated vasodilation in small mesenteric arteries of *db/db* diabetic mice was impaired compared with their lean littermates, suggesting that reduced function of adiponectin may contribute to the endothelial dysfunction observed in *db/db* diabetic mice (27). Notably, impaired vasodilation in response to adiponectin is associated with decreased APPL1 expression in diabetic arteries (Fig. 7). This novel finding raises the possibility that the decreased APPL1 expression might be causally associated with impaired vasodilation and endothelial dysfunction in diabetes. However, our current study cannot explain why APPL1 expression is selectively decreased in small mesenteric arteries of *db/db* diabetic mice. Our in vitro data showed that neither acute nor long-term treatment of HUVECs with high concentrations of glucose and/or insulin had any effect on APPL1 expression (K.K.Y.C. and A.X., unpublished observations), suggesting that hyperinsulinemia and hyperglycemia may not be direct contributors to the decreased APPL1 expression in small mesenteric arteries of *db/db* diabetic mice. Further studies are warranted to investigate the detailed molecular events that control APPL1 gene expression under various pathophysiological conditions.

In summary, the current study provides novel evidence demonstrating that APPL1 acts as an immediate downstream effector of adiponectin receptors to mediate adiponectin-induced phosphorylation of AMPK at Thr^{172} and eNOS at Ser^{1177}, and association of eNOS with HSP90, which collectively lead to enhanced NO production in endothelial cells. Whether APPL1 is also involved in other endothelial actions of adiponectin, such as protection of apoptosis (42), modulation of cytokine production (43), and alleviation of oxidative stress (44), warrants further investigation in future studies.

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