Adropin Is a Novel Regulator of Endothelial Function

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Background—Adropin is a recently identified protein that has been implicated in the maintenance of energy homeostasis and insulin resistance. Because vascular function and insulin sensitivity are closely related, we hypothesized that adropin may also exert direct effects on the endothelium.

Methods and Results—In vitro cell culture models were partnered with an in vivo murine injury model to determine the potential vascular effects of adropin. Adropin was expressed in human umbilical vein and coronary artery endothelial cells (ECs). Adropin-treated endothelial cells exhibited greater proliferation, migration and capillary-like tube formation and less permeability and tumor necrosis factor-α–induced apoptosis. In keeping with a vascular protective effect, adropin stimulated Akt Ser173 and endothelial nitric oxide (NO) synthase Ser1177 phosphorylation. The former was abrogated in the presence of the phosphatidylinositol 3-kinase inhibitor LY294002, whereas the latter was attenuated by LY294002 and by mitogen-activated protein kinase kinase 1 inhibition with PD98059. Together, these findings suggest that adropin regulates NO bioavailability and events via the phosphatidylinositol 3-kinase-Akt and extracellular signal regulated kinase 1/2 signaling pathways. Adropin markedly upregulated vascular endothelial growth factor receptor-2 (VEGFR2) transcript and protein levels, and in VEGFR2-silenced endothelial cells, adropin failed to induce phosphorylation of endothelial NO synthase, Akt, and extracellular signal regulated kinase 1/2, supporting VEGFR2 as an upstream target of adropin-mediated endothelial NO synthase activation. Last, adropin improved murine limb perfusion and elevated capillary density following induction of hindlimb ischemia.

Conclusions—We report a potential endothelial protective role of adropin that is likely mediated via upregulation of endothelial NO synthase expression through the VEGFR2-phosphatidylinositol 3-kinase-Akt and VEGFR2-extracellular signal regulated kinase 1/2 pathways. Adropin represents a novel target to limit diseases characterized by endothelial dysfunction in addition to its favorable metabolic profile. (Circulation. 2010;122[suppl 1]:S185–S192.)

Key Words: adropin ■ angiogenesis ■ endothelium ■ nitric oxide

The endothelium plays a central role in the maintenance of vascular homeostasis, and impaired endothelial function contributes to the development and progression of diverse cardiovascular, inflammatory, metabolic, infectious, and renal diseases, of which atherothrombosis has the largest clinical impact.1 Endothelial cell (EC) homeostasis is maintained in part through the synthesis of nitric oxide (NO), from the precursor L-arginine, under the influence of endothelial NO synthase (eNOS). Aside from exerting several critical antiinflammatory, antithrombotic, and antiatherosclerotic roles within blood vessels, NO also promotes postnatal angiogenesis and reparative vasculogenesis. Furthermore, there is good evidence that NO bioavailability serves an important role in metabolic regulation and insulin sensitivity.2–4

The evidence so far accrued suggests that paracrine and neuroendocrine factors, such as adiponectin, leptin, resistin, and visfatin, beyond their roles in metabolic regulation and energy expenditure, may be important modulators of endothelial function and atherosclerosis. Derangements in their production and/or action may thus underlie the development of not only insulin resistance but also endothelial dysfunction.5–16

Adropin is a recently identified protein that appears to participate in the maintenance of energy homeostasis and insulin response.17 It is encoded by the Energy Homeostasis...
Associated gene (Enho) that is expressed in the liver and the brain. In diet-induced obese mice, transgenic overexpression or systemic administration of adropin markedly attenuated insulin resistance and glucose intolerance, both key components of the metabolic stress response.

Because vascular function and insulin sensitivity are closely related, we hypothesized that adropin may also exert direct effects on the endothelium. This line of inquiry would position this novel protein as a potential gatekeeper of vascular health and may consequently implicate it as an important component of cardiometabolic diseases.

**Methods**

**Materials**

Human ECs were from Cambrex. Recombinant adropin (34-76) (human/rat/mouse), from Phoenix Biosciences, was dissolved in water. SU1498 ((E)-3-(3, 5-diisopropyl-4-hydroxyphenyl)-2-((3-phenyl-propyl)amino-carbonyl)acrylonitrile) was from Biosource.

Mock small interfering RNA (siRNA) and the siRNA siKDR\#7823 that is directed against human vascular endothelial growth factor receptor-2 (VEGFR2) were from Ambion. Rabbit polyclonal antibodies against phospho-eNOS (Ser1177), Akt, phospho-Akt (Ser473), extracellular signal regulated kinase (ERK1/2), phospho-ERK 1/2, and Bax were from Cell Signaling. Monoclonal antibodies against extracellular signal regulated kinase (ERK1/2), phospho-ERK1/2, Bcl2, Bax, and actin were from Cell Signaling. Rabbit polyclonal VEGF was from Calbiochem. Rhodamine conjugated BS-1 isoelectin-B4 from Bandeiraea simplicifolia was from Vector Laboratories. All other chemicals were from Sigma.

**Cell Culture**

Human umbilical vein ECs (HUVECs) were cultured, up to passage 7, in FBS supplemented MCDB-131 complete medium (VEC Technologies). Human coronary artery ECs maintained in endothelial growth medium-2 (Cambrex) were used at passages 2 or 3.

**Transfection**

VEGFR2 gene silencing in the reported studies was achieved by incubating HUVECs for 24 hours with a siRNA-Opti-MEM (Invitrogen)-siPORT NeoFX (Ambion) cocktail. Preliminary optimization studies indicated that transfection efficiency was similar regardless of whether HUVECs were treated for 24 or 48 hours with the VEGFR2 transfection medium (supplemental Figure I, available online at http://circ.ahajournals.org). VEGFR2 silencing with another siRNA siKDR\#7824 was similar to that observed with siKDR\#7823 (supplemental Figure I).

**RNA Analysis**

Total RNA, extracted with the RNasy Plus Mini kit, was reverse transcribed and subjected to real-time PCR (Applied Biosystems PRISM 7900HT). Oligonucleotide primers for human adropin were 5’-ATTGAGCCGACTTCACTGGC-3’ and 5’-CTGGAGTTGGGACCTGGAT-3’. Primers for GAPDH were 5’-CACCAGGGCTGCTTTTGAT-3’ and 5’-CCTTGACGGTGCCATGGATTG-3’.

**EC Function**

Adropin-treated (10 or 25 ng/mL) HUVECs were incubated with 10 mmol/L bromodeoxyuridine for 18 hours. HUVEC proliferation, as defined by bromodeoxyuridine incorporation, was determined with an enzyme-linked immunosorbent assay (Roche Diagnostic). Migration was monitored in adropin-treated (10 ng/mL) HUVECs plated in modified Boyden chambers (BD Bioscience). Migrating cells were fixed, stained with a Diff-Quik Stain (Dade Behring), and quantified in 3 random fields 4 hours later. In some cases, HUVECs were treated with 0.1 mmol/L N(G)-nitro-L-arginine methyl ester (L-NAME) for 24 hours prior to adropin exposure. Capillary-like tube formation was assessed in HUVECs treated for 4 hours with 10 ng/mL adropin. Quantification was performed with the National Institutes of Health (NIH) ImageJ analysis software in 5 random microscopic fields.

To study EC permeability, adropin-treated (10 or 25 ng/mL) HUVECs were allowed to establish a monolayer in collagen-coated Transwell inserts (Corning), before the medium in the upper compartment was replaced with a fluorescein isothiocyanate-BSA tracer solution (250 mg/mL; Invitrogen), and that in the lower compartment was replaced with 1.5 mL of fresh medium. Permeability was measured 12 hours later by fluorescein isothiocyanate-BSA transmigration across the cell monolayer.

**NO Production**

Nitrite and nitrate levels served as surrogates of NO production. Total nitrite concentrations were spectrophotometrically measured at 540 nm with the Griess reagent and sample nitrite levels calculated against a standard curve prepared with NaNO₂.

**Flow Cytometry**

Apoptotic HUVEC death was assessed by flow cytometry following tumor necrosis factor-\(\alpha\) treatment (10 ng/mL, 18 hours). HUVECs were washed in PBS then stained with Annexin V-fluorescein isothiocyanate and propidium iodide. Fluoresceins were excited at 488 nm and emissions detected at 512 and 543 nm on a Beckman Coulter Cytomics FC500 system.

**Western Blot Analyses**

Proteins from whole-cell lysates of HUVECs were separated on 4 to 12% Tris-glycine gels and transferred to nitrocellulose membranes. Complete and uniform transfer was confirmed by Ponceau S staining. Membranes were probed with antibodies directed against VEGFR2, (phospho-)eNOS, (phospho-)Akt, (phospho-)ERK1/2, Bcl2, Bax, and actin followed by incubation with appropriate horseradish peroxidase-associated secondary antibodies before signals were visualized by enhanced chemiluminescence (Amer sham Bioscience). When necessary, HUVECs were incubated with 10 mmol/L SU1498 for 15 minutes before adropin was added to the culture medium. In other cases, measurements of phosphorylated-eNOS, phosphorylated-Akt, and phosphorylated-ERK1/2 were performed on the lysates of HUVECs that had been treated...
with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (50 μmol/L) for 2 hours or the mitogen-activated protein kinase kinase 1 inhibitor PD98059 (50 μmol/L) for 30 minutes. LY294002 and PD98059 were dissolved in dimethyl sulfoxide (DMSO) (final concentration 0.1% DMSO vol/vol), which on its own had no effect on any of the outcome measurements. All cells, regardless of pretreatment, were stimulated with adropin (10 ng/mL) for 0 to 60 minutes.

**Animals**

All animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the institutional Animal Care Committee. Male Balb/c and homozygous NOS 3, EC knockout Nos3tm1Unc/J mice were purchased from The Jackson Laboratory.

**Mouse Hindlimb Ischemia**

Unilateral hindlimb ischemia was performed on 8-week-old male Balb/c mice or Nos3tm1Unc/J mice. Briefly, the skin overlying the abdomen and left limb was incised to allow ligations of the proximal end of the femoral artery and the distal portion of the saphenous artery before complete excision of the femoral artery and its attached side branches. Immediately following surgery, the adropin plasmid (500 μg) or the null plasmid was injected IM at 3 locations of the adductor muscle and at 2 locations of the gastrocnemius muscle of one leg with the other leg serving as control. Adropin gene expression in these muscles was confirmed by real-time PCR. Perfusion recovery over the next 28 days was monitored by laser Doppler flow imaging using the Moor Laser Doppler Imager system and analyzed with proprietary software (version 5.0; Moor Instruments). The region of interest that covers each hindlimb area was standardized for recording of mean perfusion and variability in

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**Figure 2.** Adropin stimulates angiogenic-like events in HUVECs. A, Proliferation and migration in HUVECs incubated with vehicle, adropin (10 or 25 ng/mL), adropin (10 ng/mL), and L-NAME or VEGF (50 ng/mL). Data expressed as a percentage of the vehicle group; n=5. B, Proliferation and migration in HUVECs transfected with mock siRNA, mock siRNA and adropin (10 ng/mL) or VEGFR2 siRNA and adropin (10 ng/mL). Data expressed as a percentage of the mock siRNA-treated group; n=5. C, Representative micrographs; and D, quantification of tube-forming activity in HUVECs 4 hours after initiating vehicle, adropin (10 ng/mL), or VEGF (50 ng/mL) treatment; n=5. E, Permeability of HUVECs treated with vehicle or adropin (10 or 25 ng/mL); n=6. *P<0.05 vs adropin vehicle group, †P<0.05 vs corresponding adropin alone group, ‡P<0.05 vs mock siRNA-treated group, §P<0.05 vs mock siRNA and adropin treated group.
the region of interests and calculation of the perfusion difference and perfusion ratio between the ischemic limb and the nonischemic control limb. On day 28, gastrocnemius muscles isolated from the ischemic limbs were embedded and frozen in Tissue-Tek O.C.T. for cryosectioning (7 μm). The density of rhodamine conjugated BS-I isolectin-B4-stained capillaries was determined with the NIH ImageJ software.

**Statistical Analyses**

Data are presented as mean±SE. Differences between 2 groups were determined with the Student’s t test. Comparisons of intergroup means were performed by ANOVA followed by the Student’s t test. Repeated measures ANOVA was used to compare blood flow recovery in the ischemic versus nonischemic hindlimb. Differences were considered significant at P<0.05.

**Results**

**Adropin Is Constitutively Expressed in ECs and Regulates EC Function and Apoptosis**

Transcript (mRNA) analyses revealed that adropin is expressed in HUVECs and human coronary artery ECs (Figure 1). As shown in Figure 2A through 2D, greater proliferation, migration, and capillary-like tube formation were observed in adropin-treated HUVECs compared with the vehicle-treated group. These responses were comparable with those elicited by exogenous VEGF (50 ng/mL) and were suppressed in HUVECs treated with L-NAME or the VEGFR2 siRNA. Adropin also attenuated HUVEC permeability to BSA, a finding suggestive of a role of adropin in improving endothelial barrier function (Figure 2E). Via flow cytometry, we further noted that adropin abrogated TNF-α-induced apoptosis (Figure 3A), with associated increases in the TNF-α-induced apoptotic Bcl2/Bax ratio (Figure 3B).19,20 Taken together, these data indicate a novel endothelial protective effect of adropin.

**Adropin Increases NO Release and Activates eNOS via the PI3K-Akt and ERK1/2 Pathways**

Adropin promoted NO release from HUVECs in a time-dependent manner (Figure 4A). Phosphorylation of eNOS at Ser1177 occurred as early as 5 minutes after adropin stimulation and rose in a time-dependent manner with protein levels of phospho-eNOS peaking after 30 minutes of incubation (Figure 4B). Parallel experiments indicated that eNOS phosphorylation at Thr495 was unaffected by adropin (data not shown). Adropin-stimulated eNOS phosphorylation at Ser1177 was inhibited in the presence of LY294002 (Figure 4C), further suggesting that adropin-induced eNOS activation is at least in part PI3K dependent. Although total Akt levels were unaffected by adropin, Ser473 Akt phosphorylation was evident within 5 minutes of incubation with adropin, optimal at the 15 minutes time point, and still present after 60 minutes of stimulation (Figure 5A). LY294002 had no effect on total HUVEC Akt protein levels in the presence of adropin but profoundly attenuated adropin-evoked Ser473 Akt phosphorylation (Figure 5B), corroborating PI3K as an upstream effector of adropin-mediated Akt activation at Ser473.

**Adropin Activates VEGFR2**

VEGFR2, a receptor of the VEGF-kinase ligand/receptor signaling system, is preferentially expressed in ECs and regulates endothelial function and angiogenesis via activation of PI3K and ERK1/2. VEGFR2 transcript and protein expressions in adropin-treated HUVECs were markedly upregulated (Figure 7). Furthermore, adropin-elicited eNOS, Akt, and ERK1/2 phosphorylation were significantly impaired in VEGFR2-silenced HUVECs and HUVECs that were incubated with the VEGFR2 inhibitor PD98059. These findings collectively suggest that adropin may be a trigger of the ERK1/2 signaling pathway in ECs.
inhibitor SU1498, suggesting that VEGFR2 is a target of adropin, with resultant downstream effects on PI3K- and ERK1/2-mediated eNOS activation (Figure 8).

Adropin Modulates Neovascularization In Vivo
Relative to the null plasmid-treated group, Balb/c mice that received the adropin plasmid demonstrated improved limb perfusion following hindlimb ischemia (Figure 9A and 9B), an effect that corresponded with higher capillary density in the ischemic gastrocnemius muscle (Figure 9C). Additionally, gastrocnemius muscles isolated from adropin-treated mice 28 days after femoral ligation exhibited significantly higher VEGFR2 protein levels and greater eNOS, Akt, and ERK1/2 phosphorylation relative to those from mice administered the null plasmid (Figure 9D). Notably, no appreciable recovery of blood flow was observed following hindlimb ischemia in Nos3tm1Unc/J mice similarly treated with the adropin plasmid (Figure 9E).

Discussion
We provide the first evidence suggesting a vascular effect of adropin. In the present study, we hypothesized that adropin may directly upregulate eNOS expression, and via this mechanism may serve to regulate EC function. We report the novel observation that adropin markedly elevates eNOS protein levels and mRNA expression in both HUVECs and coronary artery ECs. eNOS bioavailability is regulated by at least 3 different mechanisms, including transcriptional up-regulation of eNOS, posttranscriptional activation of eNOS, and reduction of reactive oxygen species-mediated breakdown of NO. We demonstrate that adropin affects endothelial NO synthesis by posttranscriptional stimulation of eNOS protein. Our results also clearly indicate that adropin activates protein kinase Akt, which leads to posttranscriptional activation of eNOS via phosphorylation of the amino acid Ser1177. Additionally, that specific use of the PI3K inhibitor LY294002 reduced adropin-induced Akt phosphorylation indicates that the PI3K-Akt signaling pathway is activated by adropin. The mitogen-activated protein kinase 1 antagonist PD98059 reduced adropin-induced ERK1/2 phosphorylation in a PI3K- and ERK1/2-dependent manner. HUVECs were incubated with 10 ng/mL adropin for up to 60 minutes. A, HUVEC media NO levels; n=5, *P<0.05 vs 0 minutes group. B, Representative Western blot analyses and densitometric quantification for HUVEC (phospho-)eNOS levels; n=5, *P<0.05 vs 0 minutes group. C and D, Representative Western blot analyses for HUVEC (phospho-)eNOS levels following (C) a 2-hour incubation with the PI3K inhibitor LY294002 (50 μmol/L) or (D) a 30-minutes treatment with the mitogen-activated protein kinase 1 inhibitor PD98059 (50 μmol/L).
phorylation, implying that adropin also exerts its effects via ERK1/2. Together, these data suggest that adropin is a novel regulator of eNOS via these dual pathways. In keeping with an effect to upregulate eNOS production and eNOS-mediated events, adropin promoted critical EC function indices, such as proliferation, migration, and capillary-like tube formation and diminished permeability and apoptosis, as well as increased activation of eNOS, Akt, and ERK1/2 and improved angiogenic potential in vivo. This line of thought is further supported by the absence of improved flow following hindlimb ischemia in adropin-treated Nos3tm1Unc/J mice.

VEGFR2, a tyrosine kinase receptor, is activated by VEGF and is upstream of the PI3K-Akt and ERK1/2 pathways in modulating EC survival and function. Our data indicate that adropin potently upregulates VEGFR2 in ECs and that gene silencing of VEGFR2 significantly impaired the effects adropin had on Akt, ERK1/2, and eNOS phosphorylation. Collectively, these data support the notion that adropin may modulate eNOS bioactivity in part through upstream activation of VEGFR2 with resultant activation of the PI3K-Akt and ERK1/2 pathways. That VEGFR2 silencing and pharmacological inhibition of VEGFR2 incompletely inhibited Akt and ERK1/2 phosphorylation and eNOS activation suggests that aside from VEGFR2, other upstream triggers of PI3K- and ERK1/2-mediated events exist and participate in this cascade.

In addition to mediating eNOS activation, Akt may engage several NO-independent pathways, including GSK3, p21/p27, EDG-1, and FOXO, which serve to regulate endothelial survival and function. Akt also plays a direct role in the mobilization of bone marrow-derived endothelial progenitor cells, which are essential in postnatal neovascularization. Therefore, the ability of adropin to augment Akt phosphorylation may uncover multiple NO-dependent and NO-independent signaling pathways that serve to improve vascular function.

Adropin was identified in 2008 as a secreted protein critically involved in energy homeostasis, metabolic adaptation to macronutrients, and modulation of insulin sensitivity and diabesity. Overexpression or systemic administration of adropin in diet-induced obese mice resulted in marked improvement in insulin sensitivity, a reduction in diabetes, and weight loss. Our data suggest that, in addition to these important metabolic effects, adropin may possess nonmetabolic properties that include regulation of eNOS bioactivity and endothelial function. Because endothelial function plays an important role in the development and progression of atherothrombosis, our findings suggest that adropin may be a novel target to limit vascular diseases, in parallel with the documented effects on metabolic modulation.

A growing body of evidence has positioned eNOS activation, and endothelial function, as mediators of whole-body insulin sensitivity. Upregulation of eNOS with resultant improvement in skeletal muscle blood flow may
in turn improve insulin sensitivity. Several paracrine and neuroendocrine factors that regulate metabolic responses and energy homeostasis have also been demonstrated to exhibit distinct effects on endothelial function and vascular tone. For example, adiponectin, a protein critically involved in insulin sensitivity, has been demonstrated to have potent effects of modulate endothelial function, and retard atherosclerosis.\textsuperscript{14,15} Likewise, resistin, a molecule known to inhibit insulin sensitivity, has deleterious effects on endothelial function.\textsuperscript{10,16}

In summary, adropin, a novel secreted energy homeostasis protein, has unique effects to regulate EC function, via upregulating eNOS expression through the VEGFR2-PI3K-Akt and VEGFR2-ERK1/2 pathways. Further, to solidifying the concept of the metabolic-vascular axis, these data suggest that in addition to the powerful effects to limit diabetes and obesity, adropin-based therapies may have the potential to improve endothelial function, promote angiogenesis, and retard atherothrombosis.

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\textbf{Disclosures}

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Optimization of VEGFR2 Gene Silencing. HUVECs were treated for 24h or 48h with mock siRNA, siRNA 7823 or siRNA 7824 prior to determination of VEGFR2 A, transcript and B, protein levels. $n=4$; *$P<0.05$ vs. mock siRNA group.