Adiponectin Stimulates Angiogenesis in Response to Tissue Ischemia through Stimulation of AMP-activated Protein Kinase Signaling***

Obesity is a risk factor for the development of cardiovascular diseases that are associated with impaired angiogenesis. Adiponectin is an adipocyte-specific adipocytokine with anti-atherogenic and anti-diabetic properties, and its plasma levels are reduced in association with obesity-linked diseases. Here, we investigated whether adiponectin regulates angiogenesis in response to tissue ischemia using adiponectin knock-out (KO) mice. Angiogenic repair of ischemic hind limbs was impaired in adiponectin-KO mice compared with wild-type (WT) mice as evaluated by laser Doppler flow method and capillary density analyses. Adenovirus-mediated supplement of adiponectin accelerated angiogenic repair in both adiponectin-KO and WT mice. Intramuscular injection of an adenovirus encoding dominant-negative AMP-activated kinase diminished the improvement in limb perfusion seen in WT mice and abolished the adiponectin-induced enhancement of perfusion. These data indicate that adiponectin can function to stimulate angiogenesis in response to ischemic stress by promoting AMP-activated kinase signaling. Therefore, adiponectin may be useful in the treatment for obesity-related vascular deficiency diseases.

Obesity contributes to the development of metabolic syndrome and type 2 diabetes (1). Both of these conditions are associated with microvascular rarefaction and reduced collateralization in ischemic tissues (2–5). These circulatory changes can lead to cardiac dysfunction, increased vulnerability to ischemic injury, and impaired wound healing. Presumably obesity-related disorders contribute to the pathogenesis of vascular complications by perturbing the levels of angiogenesis regulatory factors or by decreasing the responsiveness of tissues to proangiogenic agents.

Adiponectin/ACRP30 is a circulating adipocyte-derived cytokine that is down-regulated in association with obesity-linked diseases including coronary artery diseases (6, 7), metabolic syndrome (8), and type 2 diabetes (9). Adiponectin knock-out (KO) mice exhibit diet-induced insulin resistance that is associated with diminished insulin receptor substrate 1-mediated phosphatidylinositol 3-kinase signaling in muscle (10). These mice also display increased intimal hyperplasia in response to acute injury (11) and impaired endothelium-dependent vasodilation (12). Conversely, adiponectin overexpression reduces atherosclerotic lesions in a mouse model of atherosclerosis (13) and has anti-inflammatory effects on the vasculature (6, 14, 15). Collectively, these data suggest that adiponectin acts as a biologically relevant modulator of vascular remodeling with anti-atherogenic and anti-diabetic properties.

In the present study, we investigated whether adiponectin regulates the angiogenic process in vivo employing the hind limb model of ischemia-induced angiogenesis in mice. The ability of adiponectin to regulate angiogenesis was investigated with loss- and gain-of-function genetic manipulations using adiponectin-KO mice and an adenoviral vector that expresses adiponectin. Our observations indicate that adiponectin promotes angiogenesis in response to tissue ischemia via the activation of myogenic AMPK-dependent signaling.

EXPERIMENTAL PROCEDURES

Materials—Phospho-AMPK (Thr-172) was purchased from Cell Signaling Technology (Beverly, MA). Tubulin antibody was purchased from Oncogene (Cambridge, MA). Adenovirus vectors containing the gene for β-galactosidase (Ad-βgal), full-length mouse adiponectin (Ad-APN), and dominant-negative AMPKα2 (Ad-dnAMPK) were described previously (11, 16, 17).

Mouse Model of Angiogenesis—Adiponectin-KO (APN-KO) and wild-type (WT) mice in a C57BL6 background were used for this study (10). Study protocols were approved by the Institutional Animal Care and Use Committee at Boston University. Mice at the ages of 8–11 weeks were subjected to unilateral hind limb surgery under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally). In this model, the entire left femoral artery and vein were excised surgically (18). Before surgery and on postoperative days 3, 7, 14, and 28, body weight and systolic blood pressure were determined using a tail cuff pressure analysis system in the conscious state.

Measurement of Plasma—Blood samples were collected by heart puncture from mice on postoperative day 7. Total cholesterol, high density lipoprotein cholesterol, and glucose levels were measured with enzymatic kits, and insulin levels were assayed with an enzyme immunnoassay kit (Wako Chemicals, Richmond, VA). Mouse adiponectin levels were determined with adiponectin enzyme-linked immunosorbent assay kits (Osuka Pharmaceutical Co. Ltd., Tokyo, Japan). For this measurement blood was collected from the tail vein at the time of hind

³ The abbreviations used are: KO, knock-out; dn, dominant-negative; AMPK, AMP-activated kinase; APN, adiponectin; WT, wild-type; LDIF, laser Doppler blood flow; Ad, adenovirus.

28670 This paper is available on line at http://www.jbc.org
Limb surgery, which was 3 and 10 days after the administration of the adenoviral vectors.

Laser Doppler Blood Flow Analysis—Hind limb blood flow was measured using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Devon, UK). Immediately before surgery and on postoperative days 0, 3, 7, 14, and 28, LDBF analysis was performed on legs and feet. Blood flow was displayed as changes in the laser frequency using different color pixels. After scanning, stored images were analyzed to quantify blood flow. To avoid data variations caused by ambient light and temperature, hind limb blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF.

Capillary Density Analysis—Capillary density within the thigh adductor muscle was quantified by histological analysis. Muscle samples were imbedded in OCT compound (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. Tissue slices (5 μm in thickness) were prepared, and capillary endothelial cells were identified by immunohistostaining for CD31 (PECAM-1; BD Biosciences). Fifteen randomly chosen microscopic fields from three different sections in each tissue block were examined for the presence of capillary endothelial cells for each mouse specimen. Capillary density was expressed as the number of CD31-positive features per high power field (×400) and the number of capillaries per muscle fiber.

**TABLE I**

|      | BW    | sBP  | TC    | HDL-C | PG    | IRI   | APN   |
|------|-------|------|-------|-------|-------|-------|-------|
| WT   | 29.2 ± 1.0 | 96 ± 3 | 79.0 ± 9.2 | 55.7 ± 10.1 | 137.2 ± 23.4 | 0.24 ± 0.16 | 7.53 ± 0.93 |
| APN-KO | 28.3 ± 1.3 | 97 ± 3 | 82.7 ± 6.1 | 52.7 ± 12.0 | 139.5 ± 22.1 | 0.27 ± 0.20 | <0.05 |

**FIG. 1.** Impaired angiogenic response in the ischemic hind limbs of adiponectin-KO mice. A, a low perfusion signal (dark blue) was observed in the ischemic hind limbs of APN-KO mice, whereas a high perfusion signal (white to red) was detected in WT mice on postoperative days 7, 14, and 28. B, quantitative analysis of the ischemic/nonischemic LDBF ratio in the WT (circle) and APN-KO (triangle) mice on postoperative days 7, 14, and 28 (n = 6). *, p < 0.001; **, p < 0.01; ***, p < 0.05 versus APN-KO mice.

**FIG. 2.** Reduced capillary density in ischemic APN-KO mice. A, immunostaining of ischemic tissues with anti-CD31 monoclonal antibody (brown) on postoperative day 14. B, quantitative analysis of capillary density in WT and APN-KO mice on postoperative day 14 (n = 6 in each group). Capillary density was expressed as the number of capillaries per high power field (×400, left) and capillaries per muscle fiber (right). *, p < 0.05 versus WT mice.
Adiponectin and Ischemia-induced Angiogenesis

Western Blot Analysis—Tissue samples obtained 3 days after surgery were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor mixture (Sigma). Protein content was then determined by the Bradford method (34). Proteins (50 μg) were separated with denaturing SDS 10% polyacrylamide gels. Following transfer to membranes, immunoblot analysis was performed with the indicated antibodies at a 1:1000 dilution. This was followed by incubation with a secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution. An ECL Plus Western blotting detection kit (Amersham Biosciences) was used for detection. The relative changes in phosphorylated AMPK were normalized to the tubulin signal and expressed as percent relative to control.

Adenovirus-mediated Gene Transfer—For adenovirus experiments ischemic/nonischemic LDBF ratios were examined 14 days after surgery, which is compatible with the time course of adenovirus-mediated gene expression. In some experiments, 2 × 10^6 plaque-forming units of Ad-APN or Ad-βgal were injected into the jugular vein of mice 3 days prior to being injected into the ischemic hind limb. Alternatively, 2 × 10^6 plaque-forming units of Ad-dnAMPK or Ad-βgal were injected into five different sites of adductor muscle in the ischemic limb at the same time that 2 × 10^6 plaque-forming units of Ad-APN or Ad-βgal were injected into the jugular vein of WT mice 3 days prior to surgery.

Statistical Analysis—Data are presented as mean ± S.E. Statistical analysis was performed by analysis of variance followed by Scheffe’s F test. A value of p < 0.05 was accepted as statistically significant.

RESULTS

Impaired Ischemia-induced Angiogenesis in Adiponectin-KO Mice—All mice survived after surgical induction of unilateral hind limb ischemia and appeared to be healthy during the follow-up period. Body weight and blood pressure did not differ between the two groups (Table I). No significant differences were observed in the plasma concentrations of glucose, insulin, total cholesterol, or high density lipoprotein cholesterol between APN-KO and WT mice (n = 6). Plasma adiponectin levels were not detected in APN-KO mice. Immediately after left femoral artery and vein resection, the ratio of blood flow between the ischemic and nonischemic hind limbs decreased to 0.24 ± 0.04 in WT and 0.23 ± 0.03 in APN-KO, indicating that the severity of the induced ischemia was comparable in the two groups.

Fig. 1A shows representative LDBF images of hind limb blood flow before surgery and at different time points after surgery in the WT and APN-KO mice. In WT mice, hind limb blood flow perfusion fell precipitously after surgery, remained impaired for 3 days, increased to 50–60% of the nonischemic limb by day 7, and ultimately returned to 80% of the nonischemic limb by day 28 (Fig. 1B). In contrast to WT mice, flow recovery in the APN-KO mice was impaired. Flow in APN-KO mice was significantly less than WT by the 7th day after surgery, and the flow difference persisted at each of the subsequent time points (14 and 28 days).

To investigate the extent of angiogenesis at the microcirculatory level, capillary density was measured in histologic sections harvested from the ischemic tissues. Fig. 2A shows representative photomicrographs of tissue immunostained with CD31. Quantitative analysis revealed that on postoperative day 14 the capillary density was significantly reduced in APN-KO mice compared with WT mice (Fig. 2B).

Elevated Adiponectin Levels Promote Angiogenesis in Response to Ischemia—To test whether supplementation of adiponectin could modulate ischemia-induced angiogenesis, an adenoviral vector expressing adiponectin (Ad-APN) was delivered via jugular vein at the time of hind limb surgery in WT and APN-KO mice. Plasma adiponectin levels in Ad-APN-treated WT mice increased to a level 2.0 times higher on the 3rd day and 1.3 times higher on the 10th day compared with the Ad-βgal-treated WT mice (Table II). In APN-KO mice, Ad-APN treatment restored plasma adiponectin to levels similar to those of Ad-βgal-treated WT mice on day 3, but plasma adiponectin levels fell slightly by day 10.

Ad-APN-treated WT mice showed a significant increase in limb perfusion 14 days after hind limb surgery compared with control mice that were treated with Ad-βgal (Fig. 3, A and B) (p < 0.05; n = 5). Furthermore, treatment with Ad-APN promoted hind limb perfusion in APN-KO mice to levels similar to those of WT mice (Fig. 3, A and B) (p < 0.01; n = 5). These results demonstrate that the adenovirus-mediated supplementation of adiponectin can rescue the hemodynamic deficit that...
is seen in the APN-KO mice and that overexpression of APN can promote angiogenesis in WT mice.

Role of AMPK Signaling on Ischemia-induced Angiogenesis—To examine the role of the AMPK pathway in ischemia-induced angiogenesis, Ad-dnAMPK was injected intramuscularly into the adductor muscle of the ischemic limb at the time of surgery. APN treatment stimulated AMPK phosphorylation in the ischemic adductor muscle (Fig. 4, A and B). Intramuscular injection of Ad-dnAMPK significantly reduced both basal and adiponectin-stimulated AMPK phosphorylation. Intramuscular injection of an adenoviral vector expressing dominant-negative AMPK reduced both basal and adiponectin-stimulated improvements in limb revascularization. AMPK is a stress-activated protein kinase that participates in the regulation of energy and metabolic homeostasis (19, 20). Adiponectin functions as an AMPK activator in multiple cell types including

DISCUSSION

The present study provides in vivo evidence that adiponectin plays an important role in the process of ischemia-induced angiogenesis. Angiogenic involvement was demonstrated in a well established hind limb ischemia model. Adiponectin-KO mice showed impaired recovery of limb perfusion following femoral artery and vein removal, and exogenous adiponectin could rescue the impairment in limb reperfusion. Moreover, wild-type mice supplemented with adiponectin displayed a more rapid recovery of limb perfusion compared with control mice.

The ability of adiponectin to promote angiogenesis is probably due to its ability to stimulate the AMPK-dependent signaling within muscle. Intramuscular injection of an adenoviral vector expressing dominant-negative AMPK reduced both basal and adiponectin-stimulated AMPK signaling in the adductor muscle of the ischemic hind limb. Furthermore, dominant-negative AMPK reduced both basal and adiponectin-stimulated improvements in limb revascularization. AMPK is a stress-activated protein kinase that participates in the regulation of energy and metabolic homeostasis (19, 20). Adiponectin functions as an AMPK activator in multiple cell types including...
Adiponectin and Ischemia-induced Angiogenesis

skeletal muscle, liver, adipocytes, and endothelial cells (21–25). With regard to skeletal muscle, adiponectin-induced AMPK signaling increases glucose metabolism and fatty acid oxidation and promotes insulin sensitivity (24). These conditions promote phosphatidylinositol 3-kinase-Akt signaling within muscle (10, 26), which could lead to angiogenic growth factor synthesis (27).

Adiponectin could also exert a proangiogenic effect in ischemic tissue by acting directly on the vascular endothelium. It has been shown that AMPK signaling mediates adiponectin-induced angiogenic and anti-apoptotic cellular responses in endothelial cells (21, 28). In addition, we have demonstrated that AMPK signaling in endothelial cells is essential for angiogenic cellular responses under conditions of hypoxia in vitro (17). In contrast to these findings, it has recently been reported that adiponectin inhibits tumor neovascularization through its interaction with vascular endothelial growth factor receptor 2 (28). These discrepancies may be subject to preparation-to-preparation variability. Therefore, this study was designed to analyze the role of adiponectin with the exclusive use of loss- and gain-of-function genetic manipulations. Collectively, multiple lines of evidence suggest that adiponectin is a proangiogenic regulator. This hypothesis is further supported by the observation that AMPK signaling is anti-apoptotic in a variety of cell types (31, 32) including endothelial cells (17, 28) and that AMPK-mediated endothelial nitric-oxide synthase phosphorylation (33) will favor a proangiogenic phenotype (18).

Collateral vessel development is impaired in patients and animal models with obesity-related disorders (2–5). The findings reported here suggest that hyapo adiponectinemia may contribute the vascular insufficiency that limits blood flow to distal vessels in obese individuals. Taken together, these data suggest that the exogenous supplementation of adiponectin could be beneficial treatment for obesity-related vascular disorders.

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