Overexpression of Adiponectin Receptors Potentiates the Antiinflammatory Action of Subeffective Dose of Globular Adiponectin in Vascular Endothelial Cells

Peng Zhang, Ying Wang, Yanbo Fan, Zhihui Tang, Nanping Wang

Objective—A decreased plasma level of adiponectin is associated with obesity and metabolic syndrome and correlated with endothelial dysfunction. This study aimed to investigate the regulated expression of the newly identified adiponectin receptors (AdipoR1 and 2) and their roles in the endothelial expression of intercellular adhesion molecule-1 (ICAM-1) in response to tumor necrosis factor (TNF)-α.

Methods and Results—Immunohistochemical study and quantitative RT-PCR demonstrated that globular adiponectin suppressed the TNF-α–induced ICAM-1 expression in a dose-dependent manner in mouse aorta and human umbilical vein endothelial cells (HUVECs). Adenovirus-mediated overexpression of AdipoR1 and 2 in ECs significantly enhanced the suppressive effect of a subeffective dose of adiponectin on TNF-α–induced ICAM-1 expression and NF-κB activation. Promoter reporter assays and small interfering RNA revealed that peroxisome proliferator-activated receptor-α may function as an important pathway downstream of adiponectin and its receptors. Furthermore, overexpression of AdipoRs in rat carotid arteries markedly decreased the induction of ICAM-1 in vivo.

Conclusions—We provide novel evidence that upregulation of AdipoRs in ECs potentiates the antiinflammatory effect of adiponectin; modulating adiponectin receptors may have potential therapeutic applications for cardiovascular complications associated with metabolic syndrome and diabetes. (Arterioscler Thromb Vasc Biol. 2009;29:67-74.)

Key Words: adiponectin ■ endothelium ■ adhesion molecule ■ gene expression ■ diabetes

Endothelial cells (ECs) exert critical functions in the maintenance of vascular homeostasis. Perturbation of ECs, often featured with an induced expression of proinflammatory adhesion molecules, is associated with diabetes, hypertension, and atherosclerosis. Obesity is a prevailing problem worldwide and is closely related to diabetes, dyslipidemia, hypertension, and atherosclerosis.

Adipose tissue secretes a variety of bioactive substances, called adipocytokines, to positively or negatively modulate endothelial function. Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30), is secreted exclusively by adipose tissue and is abundant in plasma. Adiponectin plays important roles in the regulation of insulin sensitivity and metabolism. Both full-length and C-terminal globular forms (gAcrp30) of adiponectin have antiinflammatory effects on the cellular components of the vascular wall. Adiponectin suppresses TNF-α–induced monocyte adhesion to ECs and expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin, adiponectin may attenuate the inflammatory response associated with atherogenesis. A decreased plasma level of adiponectin, hypoadiponectinemia, is found with obesity and metabolic syndrome and is correlated with endothelial dysfunction and the incidence of insulin resistance and atherosclerosis. Adiponectin receptor 1 and 2 (AdipoR1 and 2) mediate the insulin-sensitizing effect of adiponectin. Their deficiency leads to insulin resistance, but adenovirus–mediated overexpression of the receptors in liver ameliorates insulin resistance. AdipoR1 and 2 are expressed in liver and skeletal muscle and vascular cells such as macrophages, smooth muscle cells and ECs in vitro and in vivo, but their function in ECs remains largely unknown. Thus, we aimed to examine whether the receptors mediate antiinflammatory effect of adiponectin and, if so, investigate the molecular mechanisms and pathways involved.

Methods

Cells and Reagents

Human umbilical vein endothelial cells (HUVECs) were harvested by collagenase treatment of umbilical cord veins and cultured on plates coated with collagen. Cells were maintained in M199 supplemented with 20% fetal bovine serum (FBS), 20 mmol/L HEPES (pH 7.4), 1 ng/mL recombinant human fibroblast growth factor, and 90 μg/mL heparin and antibiotics. Bovine aortic endothelial cells...
(BAECs) were isolated from freshly harvested aortas and cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS. Recombinant TNF-α was from Becton-Dickinson. Lipopolysaccharide (LPS) was from Sigma-Aldrich. Recombinant murine globular adiponectin was from Cytolab (Rehovot, Israel). GW610742 and fenofibrate were from Cayman Chemicals.

Plasmids, Transfection, and Reporter Assay
The PPRE-TK-Luc, a luciferase reporter containing the herpes virus-thymidine kinase promoter downstream of 3 copies of PPAR-response elements, and the ICAM-1–luc reporter, containing a 445-bp upstream region of human ICAM-1 gene. The plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cell lysates were harvested at the indicated time to measure luciferase activity. The β-galactosidase activity was measured to normalize the transfection efficiency.

Gene Silencing
The small-interfering RNA (siRNA) targeting human PPAR-α was synthesized with the sense sequence of 5′-GTCCAATGCA-CTGGAACGTG. The scramble siRNA was used as a control. Annealed double-strand siRNA was transfected using Lipofectamine 2000.

Adenoviral Vectors and Infection
The cDNA clones encoding full-length coding regions of human AdipoR1 (pOTB-7, NM_015999) and AdipoR2 (pCMV-SPORT6, NM_024551), obtained from ATCC, were subcloned into the pcDNA-3.1/myc-HisB (−) vector. To generate the adenoviruses expressing adiponectin receptors (Ad-AdipoR1 and Ad-AdipoR2), the cDNA fragments containing adipoR1/2-myc were further subcloned into a shuttle plasmid pAdlox, a tet-off expression cassette, and recombined with an E1- and E3-deleted Δ5 viral DNA in CRE8 cells as previously described.18 Ad-TTA, the adenovirus expressing a tetracycline-responsive transactivator, and Ad-GFP, expressing green fluorescence protein, were as previously reported. The adenoviral vectors were plaque-purified, expanded, and purified by cesium chloride methods. Confluent HUVECs were coinfected with Ad-TTA and Ad-AdipoR1 or 2 at a combined multiplicity of infection of 20 in the presence or absence of tetracycline (Tc).

Quantitative RT-PCR and Northern Blotting
Total RNA was isolated with use of TRIzol reagent (Invitrogen). For quantitative RT-PCR (qRT-PCR), cDNA was synthesized with the use of Superscript II reverse transcriptase (Invitrogen) and oligo-(dT) primer (Promega). qRT-PCR involved use of iQ SYBR Green PCR Supermix in the DNA Engine Opticon realtime system (Bio-Rad Laboratories) with β-actin used as an internal control. The sequences of primers are as follows: adipR1 (forward) 5′-CTG CTC CCC ACA GC, (reverse) 5′-GAC AAA GCC CTC AGC GAT AG; adipR2 (forward) 5′-GGC ATG TCC CCT TCA TTA CA, (reverse) 5′-TGT GTC CAA ATG TTG CCT GT; ICAM-1 (forward) 5′-GCC TGG AGC TGT TTG AGA AC, (reverse) 5′-ACT GTG GGG TTC AAT CTC TG; β-actin (forward) 5′-ATC TGG CAC CAC ACC TTC, (reverse) 5′-AGC GTC CAG ACG CA.

Western Blotting
Protein was extracted from cells with lysis buffer supplemented with the protease inhibitor cocktail (Roche Diagnostics). Protein concentration was measured by BCA protein assay kit (Pierce). The samples were resolved on SDS-PAGE and blotted to nitrocellulose membranes. Immunoblots were reacted with primary antibodies against IxBa, and c-myc tag, or α-tubulin (Sigma-Aldrich), detected with use of horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized by the ECL chemoluminescence system (Amer sham Biosciences).

Animal Experiments
All animal procedures were approved by the Animal Care and Use Committee of Peking University Health Science Center. For ex vivo culture of mouse arteries, 12-week-old male C57BL/6 mice were killed, and the thoracic arteries were excised immediately and cultured in DMEM containing 10% FBS. After pretreatment with adiponectin or control medium for 8 hours, arterial segments were exposed to TNF-α for 24 hours. After fixation with 4% paraformaldehyde, arteries were snap-frozen in OCT embedding compound and stored at −80°C for subsequent experiments. Adenoviral infection of rat carotid arteries was performed as previously reported. Briefly, adult male Sprague-Dawley rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). The left common carotid artery was surgically exposed, and an arteriotomy was made on the external carotid artery. Fifty μL of viral solution containing Ad-tTA and Ad-AdipoR1, 2 or Ad-LacZ at a combined titer of 1×109 pfu was infused into the isolated carotid segment and remained for 15 minutes. Then, the viral solution was withdrawn, the external carotid artery ligated, and the blood flow to the common and internal carotid arteries restored. After recovery for 24 hours, LPS (2 mg/kg) was intraperitoneally injected. Rats were euthanized 24 hours later, and the common carotid arteries were removed and snap-frozen in the OCT compound for immunohistochemical study.

Immunohistochemistry
The snap-frozen vessel segments were cryosectioned. Endogenous peroxidase activity was blocked with 3% H2O2, and sections were incubated with mouse monoclonal antibody against ICAM-1 (Santa Cruz) or c-myc tag, then HRP- or rhodamine-conjugated goat antimouse secondary antibodies. Immunoreactivity was detected with the color reaction, with 3,3′-diaminobenzidine tetrahydrochloride (DAB) used as substrate, or by fluorescence microscopy. Negative controls involved use of IgG from preimmunized mice.

Statistical Analysis
Quantitative data are expressed as means±SEM. Differences were analyzed by ANOVA or Student t test. A P<0.05 was considered significant. Nonquantitative results were representative of at least 3 independent experiments.

Results
Adiponectin Inhibits TNF-α–Induced ICAM-1 Expression in a Dose-Dependent Manner
To test whether adiponectin has a dose-dependent antiinflammatory effect ex vivo, we treated the mouse aorta with various concentrations of globular adiponectin before stimulation with TNF-α. Immunohistochemistry revealed that TNF-α–induced ICAM-1 expression in vascular endothelium was suppressed by adiponectin at concentrations higher than 1 μg/mL. A similar dose-dependent action was observed at the mRNA level in HUVECs (Figure 1B). qRT-PCR results showed adiponectin lost the suppressive effect at concentrations lower than 0.25 μg/mL, which thus represented a “subeffective” dose under the conditions of this study. We next examined the effect of TNF-α on the mRNA expression of AdipoR1 and 2 in ECs. The mRNA level of AdipoR1 was relatively higher than that of AdipoR2 in HUVECs (Figure 1C). TNF-α significantly decreased the AdipoR1 expression but had no effect on AdipoR 2 expression, which indicates that TNF-α may antagonize the adiponectin action by downregulating the receptor for adiponectin. TNF-α treatment did not cause cell toxicity in HUVECs (supplemental Figure I, available online at http://atvb.ahajournals.org).
Conditional Expression of Human Adiponectin Receptors Sensitizes the Antiinflammatory Effect of Adiponectin

To examine whether the adiponectin receptors mediate the antiinflammatory effect of adiponectin and whether the effect of the subeffective dose of adiponectin could be potentiated by the upregulation of its receptors, we generated tetracycline-controlled adenoviral vectors expressing AdipoR1 and 2 genes. As shown by Northern blotting, coinfection of HUVECs with Ad-AdipoR1 or 2 and Ad-tTA in the presence or absence of Tc (0.1 μg/mL) led to the overexpression of the receptors at the mRNA level, and the expression of exogenous adiporRs was switched off in the presence of Tc (Figure 2A). Western blotting with an antic-myc tag detected the overexpression of the AdipoRs and their localization on plasma membrane (Figure 2B).

**Figure 2.** Conditional expression of adiponectin receptors 1 and 2 in ECs. HUVECs were coinfected with Ad-AdipoR1 or 2 and Ad-tTA in the presence or absence of Tc (0.1 μg/mL). After 24 hours, cells were harvested for extraction of RNA or membrane protein. A, Northern blots were probed with the 32P-labeled cDNA for human adiporR1 or 2. B, Western blots were detected with the antibody against c-myc or β-actin. Data are representative of 3 independent experiments.

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HUVECs overexpressing adiporR1 and 2 or mock infected (with Tc) were stimulated with TNF-α for 2 hours. qRT-PCR revealed the subeffective dose of adiponectin only slightly decreasing the ICAM-1 induction by TNF-α but the suppressive effect of adiponectin on ICAM-1 induction was significantly potentiated in ECs overexpressing adiporR1 or 2 (Figure 3A). A similar effect was observed with the VCAM-1
mRNA (data not shown). In addition, TNF-α–induced ICAM-1 promoter activity was significantly inhibited by adiponectin with overexpression of AdipoRs (Figure 3B), which suggests that the receptors enhanced the transcriptional suppression of ICAM-1 gene by adiponectin. Because NF-κB is the major transcription factor controlling expression of proinflammatory genes in ECs,20 we examined the protein level of IκBα and nuclear translocation of NF-κB p65. As shown by Western blotting (Figure 3C), the TNF-α–induced degradation of IκBα and nuclear translocation of p65 were prevented by low-dose adiponectin with AdipoR1 or 2 overexpression. Taken together, these data suggested that overexpression of AdipoR1 or 2 potentiated the anti-inflammatory effect of low-dose adiponectin in ECs, likely through the inhibition of NF-κB activation.

It has been previously shown that globular adiponectin inhibited NF-κB signaling through a cAMP-dependent pathway,6 and we have thus examined the potential involvement of this mechanism. We found that adenosine-3′, 5′ cyclic phosphorothioate (Rp-cAMP), a cAMP antagonist, attenuated the adipoR2-mediated suppression. However, inhibition of cAMP pathway had less effect on adipoR1-mediated suppression of NF-κB activation (supplemental Figure III). The result suggested that the overexpression of AdipoR2-enhanced antiinflammatory effect of globular adiponectin was at least partly mediated through a cAMP-dependent pathway.

Adiponectin Activates PPAR-α via a p38 Pathway in ECs

In a number of cell types, including hepatocytes and C2C12 muscle cells, adiponectin enhances the activity of PPAR-α, which has a potent antiinflammatory effect.11 Thus, we examined whether adiponectin activates PPAR-α in ECs. BAECs were used in these experiments because they are more efficiently transfected than HUVECs. PPRE-reporter assay revealed that the ligand-dependent PPAR-α activity was increased by globular adiponectin in a dose-dependent manner. Although low-dose adiponectin (0.25 μg/mL), under the basal level, was not sufficient to activate PPAR-α (Figure 4A), it significantly stimulated PPAR-α activity with overexpression of adipoR1 or 2 (Figure 4B), indicating that PPAR-α is a downstream target of adiponectin receptors.

We next examined the upstream mechanism underlying the adiponectin receptor-mediated activation of PPAR-α activity. Western blotting revealed that overexpression of adipoR1 or 2 increased the phosphorylation of p38 (Figure 4C) in ECs. Furthermore, pretreatment with SB203580, a p38 inhibitor, significantly abolished the adipoR2-mediated PPAR-α activ-

Figure 3. Overexpression of adipoR1 or 2 potentiates the anti-inflammatory effects of low-dose adiponectin. A, HUVECs were coinfected with Ad-TTA together with Ad-adipoR1 or Ad-adipoR2 in the presence (mock) or absence of Tc for 24 hours. Cells were pretreated with control or adiponectin (0.25 μg/mL) for 8 hours and stimulated with TNF-α for 2 hours. ICAM-1 mRNA level was assayed by qRT-PCR and expressed as a proportion of TNF-α induction normalized to that of β-actin.
Adiponectin activates PPAR-α via p38 pathway in ECs. A, BAECs were coinfected with Ad-tTA and Ad-PPAR-α and then transfected with PPRE-TK-luc reporter plasmid for 24 hours. Cells were treated with adiponectin at indicated concentrations for 8 hours and stimulated with fenofibrate (50 μmol/L). B, BAECs were coinfected with Ad-PPAR-α together with Ad-adipoR1 or 2 and then transfected with PPRE-TK-luc for 24 hours before stimulation with low-dose adiponectin (0.25 μg/mL).

Figure 4. (continued). In the presence of fenofibrate. Bars represent fold induction over basal control level (mean ± SEM; n=3). *P<0.05, **P<0.01. C, HUVECs were infected with Ad-adipoR1 or 2 for 24 hours. Cellular protein was immunoblotted with antibody against phosphorylated p38 or α-tubulin. D, Ad-PPAR-α–infected BAECs were transfected with PPRE-TK-luc reporter plasmid. After 24 hours, cells were pretreated with DMSO (control) or SB203580 (10 μmol/L) for 1 hour and exposed to adiponectin for 8 hours before fenofibrate treatment. Data are representative of 3 experiments (means ± SEM). *P<0.05. **P<0.01.
ECs. The level of circulating adiponectin is negatively correlated with endothelial dysfunction \(^{11}\) and the incidence of insulin resistance and atherosclerosis. \(^{12,13}\) A causative relation between hypoadiponectinemia and atherogenesis was further strengthened by evidence that globular adiponectin transgenes or adenovirus-mediated overexpression of adiponectin ameliorates atherosclerosis in apoE-deficient mice. \(^{9,10}\) which suggests that increasing the circulating level of adiponectin may have a beneficial effect on insulin sensitivity and EC function. However, recent studies also suggest that adiponectin signaling is blunted in obesity, referred to as adiponectin resistance. \(^{21,22}\) In insulin-resistant mice, adiponectin administration failed to lower glucose levels or elicit its signaling activation. \(^{23}\) The expression of AdipoR1 or 2 in ob/ob mice was significantly decreased in insulin-sensitive tissues such as skeletal muscle and adipose tissue, which was correlated with decreased adiponectin binding in the tissue, and thus led to adiponectin resistance. \(^{24,25}\) In this study, we found that TNF-\(\alpha\), a proinflammatory adipokine, significantly decreased the mRNA expression of AdipoR1 in ECs, which suggests a potential role in endothelial dysfunction and adiponectin resistance. Low-dose globular adiponectin was unable to suppress the TNF-\(\alpha\)-induced ICAM-1 expression, thus likely mimicking the proinflammatory state associated with hypoadiponectinemia commonly seen in obesity and type 2 diabetes. However, the loss of antiinflammatory effect of low-dose adiponectin was corrected by overexpression of AdipoR1 or 2 in ECs, which suggests that modulation of AdipoRs in ECs may have a therapeutic implication in the treatment of cardiovascular diseases associated with hypoadiponectinemia and adiponec- 

## Discussion

In the present study, we provide novel evidence that overexpression of the recently identified adiponectin receptors 1 and 2 potentiﬁated the antiinflammatory effect of globular adiponectin in ECs in vitro and in vivo. AdipoR-enhanced activation of PPAR-\(\alpha\) may be a downstream pathway signaling the antiinflammatory action.

Growing evidence indicates that globular adiponectin is an antidiabetic hormone in rodents by directly regulating fatty acid and glucose metabolism and a potent antiinflammatory molecule by strongly inhibiting the expression of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin in the adiponectin on the TNF-\(\alpha\) induction of ICAM-1 activation was signiﬁcantly reduced by the PPAR-\(\alpha\) siRNA, which suggests that endogenous PPAR-\(\alpha\) was indispensable for the antiinflammatory effect of adiponectin in ECs.  

### Adenovirus-Mediated Overexpression of Adiponectin Receptors Inhibited ICAM-1 Expression In Vivo

To test whether overexpression of adiponectin receptors plays an antiinflammatory role in ECs in vivo, rat aortas were adenovirally transduced to express AdipoR1 or 2, then stimulated with LPS to induce ICAM-1 expression in ECs. The expression of the exogenous AdipoRs in the endothelium was conﬁrmed by immunofluorescence staining with the anti-myc antibody. The induced expression of ICAM-1 was markedly attenuated in endothelia overexpressing either adiponectin receptor as compared with that expressing the control adenovirus (Figure 6).
mented the inhibitory effect of globular adiponectin on IkBα degradation and NF-κB nuclear translocation, which may contribute to the receptors’ antiinflammatory effect in ECs.

The signal transduction pathway mediating the metabolic and vascular effects of adiponectin is currently under intensive investigation. We found that overexpression of AdipoR1 or 2 in ECs enhanced the ligand-dependent activation of PPAR-α, previously proposed to be a downstream signaling pathway in mediating the metabolic effects of adiponectin in skeletal muscles, liver, and adipose tissues. PPAR-α has a well-known potent antiinflammatory effect in ECs. In addition, knockdown of endogenous PPAR-α expression by siRNA attenuated the suppressive effect of globular adiponectin on ICAM-1 induction. We thus propose that AdipoRs potentiate the antiinflammatory effect of globular adiponectin via enhanced PPAR-α activity. Yoon et al recently reported that p38 MAPK is involved in the activation of PPAR-α by adiponectin in muscle cells.25 We found that phosphorylation of p38, but not extracellular signal regulated kinase (ERK) and JNK (data not shown), was increased by overexpression of AdipoR1 or 2 in ECs. In addition, SB203580, a p38 MAPK inhibitor, reduced the activating effect of AdipoR2 on PPAR-α. In cardiac myocytes, PPAR-α can be phosphorylated at its N-terminal A/B domain by p38, which results in increased ligand-dependent trans activity.24 Therefore, adiponectin receptors likely enhance the antiinflammatory effect of globular adiponectin via a sequential cascade of p38/PPAR-α in ECs. Thus, our result demonstrates that PPAR-α not only serves as an important transcriptional factor in mediating the metabolic effects of adiponectin such as promoting fatty acid oxidation and glucose uptake but also has a critical role in mediating the antiinflammatory effect of globular adiponectin in ECs. Previous studies in mouse livers have shown that AdipoR1 is more tightly linked to the activation of AMPK pathway whereas AdipoR2 is mainly involved in the activation of PPAR-α.14 Our results show that overexpression of either adipoR1 or adipoR2 in ECs can enhance the ligand-activation of PPAR-α. Although overexpression of adipoR2 resulted a slightly stronger activation of PPAR-α than adipoR1, the difference between the two is not significant. The reasons for lack of a distinct specificity in terms of PPAR-α activity are still unclear. Given the previous report that adipoR2 deficiency mice result in a decrease in gene expression of adipoR1 in the liver and fat tissue,26 one possible explanation for the rather similar effect could be a potential change in the level of adipoR1 expression by the overexpression of adipoR2. In addition, a cross talk between the adiponectin receptors may also exist. Yoon et al recently demonstrated a hierarchical signaling cascade from adiponectin-activated AMPK to p38 and PPAR-α in skeletal muscle cells.23 Thus, our results may also indicate such a cross-talk between the downstream pathways of the receptors in ECs. Other signaling molecules, such as AMP-activated protein kinase and adaptor protein containing a pleckstrin homology domain, PTB domain and leucine zipper motif28 may also be implicated in the downstream pathways of AdipoRs in EC activation, future work is necessary to dissect the specific role of these individual pathways.

In conclusion, our results show that overexpression of AdipoR1 or 2 potentiated the antiinflammatory effect of low-dose adiponectin in ECs via, at least in part, enhanced ligand-dependent activation of PPAR-α. Upregulation or pharmacological activation of the adiponectin receptors may have potential therapeutic application in treatment of endothelial dysfunction associated with diabetes, obesity, and atherosclerosis.

**Sources of Funding**
This study is supported by the grants from the National Natural Science Foundation of China (#30470810, 30890041, and 30821001) and the Major National Basic Research Program of China (2006CB503906).

**Disclosures**
None.

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**References**


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Arterioscler Thromb Vasc Biol. 2009;29:67-74; originally published online November 6, 2008; doi: 10.1161/ATVBAHA.108.178061
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material.

Legends for supplemental figures

**Supplemental Fig. 1.** HUVECs were cultured in the medium containing 20%FBS and treated with TNF-α (2 ng/ml for 24 h). Cell viability was assessed using MTT method. The data represent mean ± SEM of three independent experiments.

**Supplemental Fig. 2.** HUVECs were exposed to TNF-α for indicated time. Protein samples from whose lysates were immunoblotted with antibody against IκBα and α-tubulin.

**Supplemental Fig. 3.** BAECs were infected with Ad-adipR1 or Ad-adipR2 before transfection with 5 x NF-κB-Luc reporter plasmid. Twenty four h later, cells were pretreated with or without R-p-cAMP (10 μM for 1 h) and exposed to TNF-α for 24 h. Luciferase activity was measured and normalized to β-galactosidase. Data are from 3 independent experiments and expressed as fold induction over control.*p<0.05.
Supplemental Fig. 1

Cell viability (%)

control  TNF-α
Supplemental Fig. 2

The diagram shows the expression levels of TNF-α, IκB-α, and Tubulin at different time points (0, 5, 10, 15, 20, and 30 minutes).
Supplemental Fig. 3

NF-κB luciferase activity

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* Significant difference