Adiponectin Inhibits TNF-α–Induced Vascular Inflammatory Response via Caveolin-Mediated Ceramidase Recruitment and Activation

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Running title: Anti-Inflammatory Signaling of Adiponectin

Subject codes:
[95] Endothelium/vascular type/nitric oxide
[147] Growth factors/cytokines
[190] Type 2 diabetes

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This manuscript was sent to Ali J. Marian, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

In December 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.66 days.

DOI: 10.1161/CIRCRESAHA.114.302439
ABSTRACT

**Rationale:** Anti-inflammatory and vascular protective actions of adiponectin (APN) are well-recognized. However, many fundamental questions remain unanswered.

**Objective:** The current study attempted to identify the APN receptor subtype responsible for APN’s vascular protective action, and investigate the role of ceramidase activation in APN anti-inflammatory signaling.

**Methods and Results:** APN significantly reduced TNFα-induced ICAM-1 expression and attenuated TNFα-induced oxidative/nitrative stress in HUVECs. These anti-inflammatory actions were virtually abolished by AdipoR1-, but not AdipoR2-, knockdown (KD). Treatment with APN significantly increased neutral ceramidase (nCDase) activity (3.7-fold, P<0.01). AdipoR1-KD markedly, whereas AdipoR2-KD only slightly, reduced gAPN-induced nCDase activation. More importantly, siRNA mediated nCDase-KD markedly blocked the effect of APN upon TNFα-induced ICAM-1 expression. AMPK-KD failed to block APN-induced nCDase activation, and modestly inhibited APN anti-inflammatory effect. In contrast, in Caveolin-1 knockdown (Cav1-KD) cells, >87% of APN-induced nCDase activation was lost. Whereas APN treatment failed to inhibit TNFα-induced ICAM-1 expression, treatment with S1P or SEW (S1P receptor agonist) remained effective in Cav1-KD cells. AdipoR1 and Cav1 co-localized and co-precipitated in HUVECs. APN treatment did not affect this interaction. There is weak basal Cav1/nCDase interaction, which significantly increased following APN treatment. Knockout of AdipoR1 or Cav1 abolished the inhibitory effect of APN upon leukocyte rolling and adhesion in vivo.

**Conclusion:** These results demonstrate for the first time that APN inhibits TNFα-induced inflammatory response via Cav1-mediated ceramidase recruitment and activation in an AdipoR1-dependent fashion.

**Keywords:** Adipokines, endothelial cells, inflammation, sphingolipids, vascular injury, vascular endothelial function

**Nonstandard Abbreviations and Acronyms:**

- ACC: acetyl-CoA carboxylase
- AdipoR1: adiponectin receptor 1
- AdipoR2: adiponectin receptor 2
- AdipoQ: adiponectin, C1Q and collagen domain-containing
- AMPK: AMP-activated protein kinase
- APN: adiponectin
- Cav1: caveolin-1
- COX-2: cyclooxygenase-2
- eNOS: endothelial nitric oxide synthase
- fAPN: full length adiponectin
- GAPDH: glyceraldehyde 3-phosphate dehydrogenase
- gAPN: globular adiponectin
- HMW: high molecular weight
- HUVEC: human umbilical vein endothelial cells
- ICAM-1: intercellular adhesion molecule-1
- KD: knockdown
- KO: knockout
- nCDase: neutral ceramidase
- NFkB: nuclear factor kappa beta
- RAEC: rat aortic endothelial cells
- rhTNFα: recombinant human tumor necrosis factor-α protein
- RSG: rosiglitazone
- SEW2871: a selective S1P receptor-1 agonist
- S1P: sphingosine-1-phosphate
- TRITC: tetramethyl rhodamine
INTRODUCTION

Cardiovascular complications are the leading cause of death for patients with type 2 diabetes, a disease affecting over 20 million people in the US1. The inflammatory response associated with diabetes and the resultant vascular injury initiate more severe diabetic cardiovascular complications, including atherosclerosis and ischemic heart disease2. Defining the mechanisms leading to inflammatory vascular injury in diabetes, and identifying novel therapeutic strategies capable of protecting vascular function, are therefore in great need.

Adiponectin (APN) is an adipocyte-derived cytokine. In contrast to the majority of adipokines (e.g., TNFα), which are pro-inflammatory and significantly increased in diabetic patients, APN is a potent vascular protective molecule that is markedly reduced in type 2 diabetic patients3-5. APN reduces oxidative/nitrative stress, protects endothelial cells from apoptosis, inhibits leukocyte-endothelial interaction, and decreases smooth muscle proliferation6. Two APN receptors (AdipoR1 and AdipoR2) have been cloned7. They belong to a new family of membrane receptors (the progestin and AdipoQ receptor superfamily)8-10 predicted to contain seven transmembrane domains, but are topologically distinct from GPCR. Although both AdipoR1 and AdipoR2 are expressed in vascular endothelial cells, their roles in APN-mediated anti-inflammatory and vascular protective actions have not been clarified.

Vasodilatory and vascular protective effects of APN have previously been attributed to AMP activated protein kinase (AMPK)-mediated eNOS phosphorylation and nitric oxide production11,12. However, we recently demonstrated that in the ischemic/reperfused heart, APN’s anti-oxidative/anti-nitrative effects are largely AMPK-independent13,14. Moreover, a recent study demonstrates that APN activates neutral ceramidase (nCDase) in an APN receptor-dependent but AMPK-independent fashion15. As ceramidase is a key enzyme converting ceramide, a pro-inflammatory molecule, to sphingosine-1-phosphate (S1P), an anti-inflammatory and cardiovascular protective molecule16-20, it is possible that APN’s anti-inflammatory and vascular protective effect is mediated by ceramidase activation. However, direct evidence supporting this attractive notion is currently lacking.

Therefore, the aims of the current study were to 1) identify the APN receptor subtype responsible for vascular protective action; 2) determine the role of ceramidase activation in APN-mediated anti-inflammatory signaling; and 3) investigate whether AdipoR1 functions as a ceramidase or mediates ceramidase activation through other signaling molecules.

METHODS

Cell culture and treatments.
HUVEC (Passage 2-3) were plated on six-well plates and cultured in endothelial growth medium containing 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO2. Upon 80% confluence, cells were treated with vehicle, gAPN (2 µg/ml) or fAPN (10 µg/ml). One hour after APN treatment, 10 ng/ml rhTNFα21 was added. Cells were collected 12 hours after TNFα treatment and oxidative/nitrative stress and ICAM-1 expression were determined as described in detail below.

Small interfering RNA transfection, plasmid construction, and transfection.
siRNA duplexes against Cav-122, AdipoR123, AdipoR225, AMPKα123, and nCDase24 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Universal control oligonucleotides (AllStars) from Santa Cruz served as negative control. HUVECs (80% confluent) were transfected via siIMPORTER siRNA transfection kit (Qiagen Science Inc. Benelux) per manufacturer’s protocol (final siRNA concentration: 50 nM).
**Ceramidase enzyme activity assay.**
The nCDase enzyme activity was determined as previously reported, with minor modification. Briefly, at experiment conclusion, cells were collected and washed twice with PBS. Cell pellets were resuspended in 100 μl 0.25 M sucrose solution, sonicated, and centrifuged at 15,000 g for 3 minutes. The supernatant was collected and protein concentration was determined. A 25 μl sample containing identical protein amount, 75 μl 25 mM phosphate buffer (pH 7.4), and 0.5 μl 4 mM Rbm14-12 substrate solution in ethanol (final substrate concentration 40 μM; final ethanol concentration 1%) were loaded into each well of a 96-well plate. The same incubation mixture without supernatant served as negative control. The plate was incubated at 37°C for 1 hour without agitation. The enzymatic reaction was stopped by adding 25 μl methanol and 100 μl NaIO4 (2.5 mg/ml) in 200 mM glycine/NaOH buffer (pH 10.6) to each well. The plate was placed in a dark room for 1 hour. Fluorescent intensity was quantified via SpectraMax Microplate Reader (Molecular Devices, λex 355 nm, λem 446 nm).

**Determination of superoxide and peroxynitrite content.**
Superoxide content was quantified by lucigenin enhanced luminescence, and the cellular origin of reactive oxygen species was determined by dihydroethidium staining (DHE, Molecular Probes, Carlsbad, CA) as previously described. Nitrotyrosine content, the footprint of peroxynitrite formation, was quantified by ELISA as previously described.

**Confocal immunofluorescence microscopy.**
30 minutes after vehicle or gAPN treatment, HUVEC were fixed with 4% paraformaldehyde/PBS in μ-Slide (ibidi LLC, Verona, WI) for 15 minutes followed by PBS washing. Cells were first treated with antibodies against AdipoR1, Cav1, or nCDase, followed by incubation with tetramethyl rhodamine (TRITC)-conjugated anti-rabbit IgG and Cy5-conjugated anti-goat IgG. For Ceramide and S1P staining, cells were fixed with 4% paraformaldehyde/PBA for 2 min. Cells were then washed with ddH2O (2 min) and TBS (pH7.6, 5 min). The primary antibodies were prepared with TBS solution containing 5μM of CaCl₂ and cells were incubated with antibody for 2 h at room temperature. After 10 min washing with TBS under agitation, cells were incubated with fluorescence labeled secondary antibody. Slides were visualized by a FV1000 confocal microscope with x60 oil-immersion objective lenses (Olympus, Tokyo, Japan). Fluorescent images were obtained by a digital camera and analyzed with Fluoview software (Olympus).

**Immunoblotting and Co-immunoprecipitation.**
HUVECs were lysed with cold lysis buffer. After homogenization and centrifugation, the supernatant was collected. For immunoblotting, proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were then incubated with primary antibodies and HRP-conjugated secondary antibody. The blot was developed with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL). Bands of interest were visualized by a Kodak Image Station 4000R Pro (Rochester, NY). For co-immunoprecipitation, cell lysates were pre-cleared with corresponding nonimmune IgG, and incubated together with protein A plus-Sepharose for 30 minutes at 4°C. Cleaned lysates were then incubated with 2 μg of either anti-Cav1 or anti-nCDase antibodies. Cell lysates were then incubated with protein A plus-Sepharose overnight at 4°C. Nonimmune rabbit IgG served as negative control. Protein A beads were then extensively washed with lysis buffer. Proteins were eluted from beads, and resolved by elution buffer. Samples with 2XSDS sample buffer were heated and separated by electrophoresis. After transfer to PVDF membranes, proteins were immunoblotted with anti-AdipoR1 (for Cav1/AdipoR1 interaction) or anti-Cav1 (for Cav1/ceramidase interaction) as described above.

**Intravital microscopy analysis of leukocyte rolling and adhesion.**
Leukocyte rolling and adhesion was assayed in mesenteric post-capillary venules by intravital microscopy as we previously described. In brief, mice were pretreated with gAPN (first dose: 1.0 μg/g, i.p., 24 hours before TNFα administration; second dose: 1 μg/g, i.p.). 30 min after the second dose of APN, mice were
treated with recombinant TNFα (1.0 µg/kg, i.p) for 2 hours. Following exteriorization of a loop of ileum tissue via a midline laparotomy, the ileum was placed in a temperature-controlled fluid-filled Plexiglas chamber and trans-illuminated for bright-field observation of the peri-intestinal microcirculation. The ileum and mesentery were perfused throughout the experiment with a buffered K-H solution (pH 7.4, 37°C). Three to four straight, unbranched segments of post-capillary venules with lengths of >100 µm and diameters between 25 and 40 µm were studied in each mouse using an Eclipse FN1 Microscope (Nikon Corp), and the image recorded and analyzed on a WIN XP Imaging Workstation. Leukocyte rolling was defined as the number of leukocytes rolling past a fixed point per minute; leukocyte adherence was defined as the number of leukocytes firmly adhered to 100-µm length of endothelium for at least 30 seconds. Rolling and adhesion were quantitated 4 hours following TNF-α injection. Venular blood velocity (V) was measured using the Microvessel Velocity OD-RT optical Doppler velocimeter (Circusoft Instrumentation) with corresponding software. Venular wall shear rate (γ) was calculated using the formula: $\gamma = 4.9 \times 8(V_{\text{mean}}/D)$, where D is the venule diameter.

Statistical analysis.
All values in the text and figures are presented as means±SEM of n independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Tukey correction for post-hoc test. Western blot densities were analyzed by the Kruskal-Wallis test followed by Dunn’s post-hoc test. Probabilities of 0.05 or less were considered statistically significant.

RESULTS

Anti-inflammatory, anti-oxidative and anti-nitrative effects of APN are mediated largely by AdipoR1.

Treatment of HUVEC with TNFα elicits significant inflammatory response as evidenced by upregulated intercellular adhesion molecule-1 (ICAM-1) expression (Figure 1), amplified superoxide generation, and increased nitrotyrosine formation (Figure 2). Consistent with previous reports, treatment with gAPN significantly inhibited TNFα-induced ICAM-1 expression (Figure 1B), inhibited superoxide generation (Figure 2A), and decreased nitrotyrosine formation (Figure 2C). To identify the receptor subtype(s) responsible for gAPN’s aforementioned anti-inflammatory actions, expression of AdipoR1 and AdipoR2 were genetically inhibited by siRNA (Figure 1A). Consistent with previous reports, expression level of AdipoR1 is higher than AdipoR2 in HUVEC (relative mRNA abundance [normalized against GAPDH]: AdipoR1=0.67±0.04; AdipoR2=0.31±0.03, P<0.01). This expression pattern is not altered by acute TNFα treatment (AdipoR1=0.64±0.03; AdipoR2=0.29±0.02, P<0.01 vs. vehicle-treated group). AdipoR1-KD slightly increased TNFα-induced ICAM-1 expression (Figure 1C) and superoxide generation (Figures 2B, P<0.05), and significantly increased nitrotyrosine formation (2.01±0.24 pmol/mg protein vs. 3.36±0.48 pmol/mg protein, P<0.05). AdipoR2-KD did not significantly modify the TNFα-induced inflammatory reaction without APN treatment (Figure 1D). Most importantly, in AdipoR1-KD HUVEC, the inhibitory effects of gAPN upon ICAM-1 expression (Figure 1C), superoxide generation (Figure 2B), and nitrotyrosine formation (Figure 2D) were all markedly (>87%) inhibited. In contrast, in AdipoR2-KD HUVEC, the anti-inflammatory (Figure 1D), anti-oxidative (Figure 2C) and anti-nitrative effects (Figure 2 F) of gAPN were largely retained. In a separate experiment, the influence of AdipoR1-KD and AdipoR2-KD upon the anti-inflammatory effects of full length APN (fAPN) was determined. The fAPN did not inhibit TNFα-induced inflammation as potently as gAPN (10 µg/ml fAPN achieved comparable inhibitory effect as 2 µg/ml gAPN and further increasing fAPN up to 20 µg/ml failed to achieve better inhibitory effect) (Figure 1B). The anti-inflammatory effect of fAPN (ICAM-1 expression presented in Figure 1 and oxidative/nitrative stress presented in Figure 2) was largely blocked by AdipoR1-KD (>76% inhibition, P>0.05 vs. TNFα with vehicle, Figure 1C) and modestly blocked by
AdipoR2-KD (<31% inhibition, P<0.05 vs. TNFα with vehicle Figure 1D). These results demonstrate that AdipoR1 largely mediates the anti-inflammatory effect of APN in HUVEC.

Activation of nCDase by gAPN contributes to its anti-inflammatory action.

A recent study demonstrates that APN activates nCDase, increasing its catalytic activity in several cell types, including β-cells, HEK 239 cells, and liver cells. This effect is blocked in AdipoR1 and AdipoR2 double knockout cells15. In HUVEC cells, neither TNFα nor APN (gAPN and fAPN) exhibited significant effect upon nCDase expression (Figure 3A). The ceramidase activation by gAPN is virtually abolished in AdipoR1 knockdown cells and preserved in AdipoR2 knockdown cells, indicating that gAPN increases nCDase activity in an AdipoR1-dependent fashion (Figures 3B). The ceramidase activity of fAPN is partially lost in either AdipoR1 and AdipoR2 knockdown cells, with modestly stronger inhibitory effect when AdipoR1 is genetically inhibited, a result consistent with recent report in liver cells (Figure 3B)15. To determine the role of nCDase activation in APN’s anti-inflammatory action, nCDase expression was genetically inhibited by siRNA (Figure 3A, left panel, last lane). As expected, APN failed to significantly increase nCDase activity in nCDase-knockdown HUVEC (Figure 3A, right panel). Most importantly, knockdown of nCDase markedly, although not completely, blocked the inhibitory effect of gAPN upon TNFα-induced ICAM-1 expression (Figure 3C).

Role of AMPK in APN activation of nCDase and anti-inflammatory action.

AMPK is a well-recognized downstream signaling molecule of APN32. To determine the relationship between AMPK and nCDase in relation to APN’s anti-inflammatory action, AMPK expression was genetically inhibited by siRNA (Figure 4A). As summarized in Figure 4B, transfection with siRNA against AMPKα successfully blocked AMPK-mediated ACC phosphorylation following APN treatment. However, AMPK-KD had no effect upon gAPN-induced nCDase activation (Figure 4C). Finally, AMPK-KD partially blocked the effect of gAPN upon TNFα-induced ICAM-1 expression (Figure 4D). These results demonstrate that APN activates nCDase in an AMPK-independent manner, but its anti-inflammatory effect is partially mediated by AMPK signaling.

Role of Cav1 in APN-induced nCDase activation and anti-inflammatory action.

We recently demonstrated that, by holding AdipoR1 and its downstream signaling molecules in close proximity, forming a caveolae-located APN “signalsome”, Cav3 (the primary caveolin isotype expressed in cardiomyocytes) plays an essential role in APN’s cardioprotective actions post myocardial ischemia/reperfusion33. To clarify whether Cav1 (the primary caveolin isotype expressed in endothelial cells34, 35) is required for APN activation of nCDase and anti-inflammatory action in HUVEC, Cav1 expression was downregulated by siRNA (Figure 5A, left panel). As summarized in Figure 5, Cav1-KD not only blocked APN activation of nCDase activity (Figure 5A, right panel), but also virtually abolished the inhibitory effect of APN upon TNFαINDUCED ICAM-1 expression (Figure 5B). To obtain more evidence that APN activates nCDase and inhibits inflammatory response in a Cav1-dependent manner, the effect of S1P and SEW2871 (a selective S1P receptor-1 agonist) upon TNFα-induced ICAM-1 expression was compared in WT and Cav1-KD cells. As summarized in Figure 5C, treatment with either S1P or SEW2871 significantly inhibited TNFα-induced ICAM-1 expression, and their anti-inflammatory action was not blocked by Cav1-KD. These results demonstrate that direct application of S1P, the end catalytic product of the CDase system, bypasses Cav1-dependent nCDase activation, and remains effective in blocking TNFα-induced inflammatory action. This result also supports the necessity of Cav1 for certain (such as AdipoR1 and insulin receptor) but not all membrane receptor-mediated signaling.

Cav1 interacts with AdipoR1 as well as nCDase, forming a signaling complex.

Results presented in Figure 3B and Figure 5A demonstrated that APN increases nCDase activity in an AdipoR1 and Cav1 dependent fashion. To determine whether the effect of APN on cellular levels of ceramide and S1P is also AdipoR1/Cav1 dependent, additional experiment was performed. As illustrated
in Figure 6A/B, treatment with APN significantly attenuated TNFα-induced ceramide accumulation and increased S1P level, particularly at cellular membrane. These effects were virtually abolished when either AdipoR1 or Cav1 expression was genetically inhibited, but largely preserved when AdipoR2 expression was genetically inhibited.

Cav1 regulates transmembrane signaling largely via engagement of protein-protein interaction, facilitated by the scaffold domain located within Cav1 and the caveolin binding motif located within its partner proteins\textsuperscript{36}. In a final attempt to clarify how Cav1 enables gAPN-initiated nCDase activation, two series of experiments were performed. First, the interaction between Cav1 and AdipoR1 was determined by immunofluorescent microscopy and co-immunoprecipitation. As illustrated in Figures 6C, colocalization of Cav1 and AdipoR1 was clearly observed (top panel). Cav1 knockdown did not alter AdipoR1 membrane localization (Figure 6C, low panel). Cav1 and AdipoR1 were co-immunoprecipitated in HUVEC (6D) and rat aortic endothelial cells (RAEC, Figure 6E). Treatment with APN had no significant effect upon this protein-protein interaction (Figure 6D/E). Moreover, re-expression of WT Cav1 in Cav1-KD cells restored Cav1/AdipoR1 interaction (Figure 6D/E, right panel, first lane). However, re-expression in Cav1-KD cells of a mutated Cav1, in which 5 aromatic residues within the scaffolding domain responsible for Cav1 interaction with partner proteins were converted to alanine\textsuperscript{37}, did not reestablish Cav1/AdipoR1 interaction (Figure 6D/E, right panel, second lane). Moreover, Cav1/nCDase interaction was also observed in aortic segment from mice treated with vehicle or gAPN (Figure 6F). Finally, co-immunoprecipitation identified APN/Cav1 interaction, indicating that APN binds the proposed Cav1/AdipoR1 complex (Figure 6G, upper panel). These results support basal Cav1/AdipoR1 interaction, a protein-protein interaction requisite for APN activation of nCDase.

In a separate experiment, potential Cav1/AdipoR2 interaction was determined by co-immunoprecipitation. As illustrated in Figure 6G (lower panel), interaction between Cav1/AdipoR2 is very weak. The precise mechanisms responsible for the preferential interaction between Cav1 and AdipoR1 over AdipoR2 remain unclear at the present time. One possible explanation is that AdipoR1 contains two caveolin binding motifs, of which one is located within its cytosolic region, whereas AdipoR2 contains only one caveolin binding motif, which is located within the transmembrane domain.

In second series of experiments, the interaction between Cav1 and nCDase was determined. Under basal conditions (vehicle), nCDase co-localization of Cav1 and nCDase is not robustly apparent (Figure 7A), and co-immunoprecipitation of Cav1 and nCDase is weak (7A). However, gAPN treatment significantly increased Cav1/nCDase interaction, as evidenced by clear co-localization (Figure 7A) and strong co-immunoprecipitation (Figure 7B/C). Although the weak basal Cav1/nCDase interaction is not affected by AdipoR1 knockdown, the APN-stimulated enhancement of Cav1/nCDase interaction is inhibited in AdipoR1 knockdown cells, but not altered in AdipoR2 knockdown cells (Figure 7B). The interaction pattern between Cav1/nCDase clearly differs from Cav1/AdipoR1. Specifically, strong Cav1/AdipoR1 interaction is observed during basal conditions, but is not regulated by APN. In contrast, basal Cav1/nCDase interaction is weak, and is markedly enhanced after APN treatment. These results suggest that AdipoR1-Cav1-nCDase forms a complex and their association is enhanced after APN treatment. To obtain more evidence supporting this notion, AdipoR1/nCDase interaction in the presence and absence of APN was determined. As illustrated in Figure 7D, weak AdipoR1/nCDase interaction was observed and treatment with APN significantly enhanced interaction.

**Inhibitory effect of APN upon leukocyte rolling and adhesion is AdipoR1 and Cav1 dependent.**

To validate in vitro finding in a pathologically relevant in vivo model, interaction of Cav1 and nCDase and effect of AdipoR1/AdipoR2 and Cav1 knockout upon APN inhibition of TNFα-induced leukocyte rolling and adhesion was determined using intravital microscopy. As illustrated in Figure 6E, Cav1/nCDase interaction and its enhancement were observed in aortic tissues from animals treated with vehicle or gAPN. Consistent with our previous report\textsuperscript{30}, in vivo treatment of WT mice with gAPN significantly inhibited TNFα-induced leukocyte rolling and adhesion (Figure 8). Cav1 knockout only
slightly (P>0.05) increased leukocyte rolling and adhesion after TNFα treatment, likely due to the increased basal nitric oxide production in these animals. In AdipoR1KO mice, greater leukocyte rolling and adhesion was observed after TNFα treatment (P<0.05 vs. WT). Most importantly, the inhibitory effect of APN upon TNFα-induced leukocyte rolling and adhesion observed in WT mice was markedly inhibited in AdipoR1 knockout mice (Figure 8), and completely abolished in Cav1 knockout mice (leukocyte rolling: 45±3.3 cells/100 µm with APN treatment vs. 48±3.8 cells/100 µm with vehicle, P>0.1; leukocyte adhesion: 6.4±0.4 cells/100 µm with APN vs. 6.6±0.5 cells/100 µm with vehicle, P>0.1). Moreover, opposite from those results observed in AdipoR1-KO mice, the inhibitory effect of APN upon TNFα-induced leukocyte rolling and adhesion was largely preserved in AdipoR2-KO mice (Figure 8). Leukocyte rolling was slightly decreased in AdipoR2-KO mice compared to AdipoR1-KO mice during basal conditions (before TNFα challenge), without statistically significant difference. This result is consistent with previous observations that no significant phenotype manifests in APN-KO mice, unless challenged by metabolic or inflammatory stress.

DISCUSSION

We have made three novel observations in this study. Firstly, we demonstrate that the anti-inflammatory (determined by ICAM-1 expression), anti-oxidative (determined by superoxide production), and anti-nitrative (determined by nitrotyrosine formation) actions of gAPN are largely mediated by AdipoR1. At least 3 possibilities potentially explaining the superior function of AdipoR1 over AdipoR2 in the vascular system exist. It has been previously demonstrated that AdipoR1 has greater gAPN binding affinity than AdipoR238, 39. However, our results indicating fAPN (which has comparable binding affinity to both AdipoR1 and AdipoR2) exhibits an anti-inflammatory effect with stronger dependency upon AdipoR1 argues against this possibility. A more likely explanation concerns prevalence. As AdipoR1 is the dominant APN receptor isotype expressed in vascular tissue39, it stands to reason AdipoR1 would be chiefly responsible for the biological effects of APN within the vascular system. Another possibility involves isotype-specific intracellular signaling. It is well-recognized that membrane receptors possessing multiple subtypes (such as TNFα and β-adrenergic receptors) initiate different or even opposite intracellular signaling through different subtype activation. It is thus possible that AdipoR1 and AdipoR2 may activate different intracellular signaling pathways, with anti-inflammatory, anti-oxidative, and anti-nitrative signaling preferentially mediated by AdipoR1. Regardless of which possibility is true, the current study strongly suggests that although interventions activating AdipoR2 may better regulate hepatic metabolism, molecules strongly activating AdipoR1 (such as the globular domain of APN) may have greater therapeutic efficacy reducing vascular injury, particularly from inflammatory changes. This conclusion is further supported by our previous study demonstrating that increased cardiomyocyte APN production and resultant cardiac protection as a result of RSG treatment is primarily mediated by AdipoR1 activation40.

Secondly, we demonstrate that gAPN activates HUVEC nCDase in an AMPK-independent fashion and nCDase activation significantly contributes to APN’s anti-inflammatory function. In recent years, the biological activities of adiponectin have been extensively investigated. Four major functions (including metabolism-regulatory, anti-inflammatory, vasculoprotective, and cardioprotective effects) have been identified41. Among multiple intracellular molecules activated after APN-binding of its specific membrane receptors, AMPK is the molecule most intensively investigated, and is generally accepted as the most important intracellular signaling molecule mediating APN biological functions32. However, accumulating evidence suggests that the degree of AMPK involvement in biological regulation of APN is dependent upon organ and disease42. Specifically, pharmacological inhibition of AMPK activity or genetic inhibition of AMPK expression virtually abolishes the central metabolic actions of APN43, 44, indicating that AMPK plays an essential role in hepatic metabolic regulation of APN. However, we13 and others45 have recently
demonstrated that the anti-ischemic/cardioprotective effects of APN are largely AMPK-independent, and involve the COX-2 and NFκB-mediated signaling pathways. Moreover, a recent elegant study reported that APN exerts anti-apoptotic and cellular protective actions in an AMPK-independent, nCDase-dependent manner\textsuperscript{15}. Our current study demonstrates that unlike APN’s metabolic and anti-apoptotic actions (largely AMPK-dependent or largely AMPK-independent), APN’s anti-inflammatory function is mediated by both the traditional AMPK pathway and the newly identified AMPK-independent nCDase activation pathway.

Thirdly, and most importantly, we provide the first direct evidence that Cav1 plays an essential role in AdipoR1-mediated nCDase activation and the anti-inflammatory actions of APN. At least two possibilities exist that may explain the necessity of Cav1 in APN vascular signaling. First, as suggested by a recent experimental study, AdipoR1 may exert nCDase activity and catalyze the ceramide reaction. Since many co-factors required for enzymatic reactions enrich in caveolae, Cav1 loss may destroy the local environment optimized for nCDase catalytic reactions. The second and more likely explanation is that binding of APN to AdipoR1 recruits nCDase to the Cav1/AdipoR1 complex, facilitating their activation. This possibility is supported by the following 4 experimental evidences: 1) Cav1 colocalizes and co-immunoprecipitates with AdipoR1, suggesting these two proteins interact with each other; 2) Mutation of 5 aromatic residues within the scaffolding domain known to be responsible for Cav1 interaction with its partner proteins not only inhibited Cav1/AdipoR1 interaction but also blocked APN activation of nCDase; 3) Cav1 interacts with nCDase in different fashion than AdipoR1. Whereas the Cav1/AdipoR1 interaction appears static, the Cav1/nCDase interface is dynamically regulated in an AdipoR1-dependent fashion; 4) Treatment with APN significantly brought Cav1/nCDase in closer proximity to each other. Scherer and colleagues previously reported that mice overexpressing APN displays elevated Cav1 levels in adipocyte\textsuperscript{46} and that Cav1 knockout significantly reduces plasma APN levels\textsuperscript{47}. Collectively, currently available experimental evidence supports that Cav1 plays essential roles in APN production as well as APN transmembrane signaling.

Cav1 is an essential molecule in EC caveolae formation. The role of Cav1 in atherosclerosis development is extremely complex. Either pro- and anti-atherosclerotic effects have been reported, depending upon cell types and disease development stage investigated\textsuperscript{36, 48}. Although the ‘dark’ mechanisms of Cav1 promoting atherosclerosis have been extensively investigated, the ‘bright’ side mechanisms of Cav1 preventing atherosclerosis remain largely unknown. Our current experiments demonstrated Cav1 interacts with AdipoR1, facilitating nCDase recruitment/activation upon APN binding, enabling the anti-inflammatory signaling of APN.

Two limitations exist in the current study. First, we have provided clear evidence that APN activation of AdipoR1 increases nCDase interaction with Cav1. However, how activation of AdipoR1 by APN increases Cav1/nCDase interaction remains unclear. Since AdipoR1 directly interacts with Cav1, it is possible that APN binding to AdipoR1 may promote Cav1 conformational change as that caused by other Cav1 interacting molecule\textsuperscript{49}, thus increasing its affinity with nCDase. This intriguing possibility will be deeply investigated in our future studies. Second, we have demonstrated that the anti-inflammatory/anti-oxidative/anti-nitrative actions of APN are largely mediated by AdipoR1, not AdipoR2. In addition to these two evolutionarily acquired specific receptors, other cell surface binding proteins (such as T-cadherin) “tether” high molecular weight (HMW) isoforms of APN to the cell surface, possibly facilitating APN/AdipoR interaction\textsuperscript{50}. The role of these proteins in APN’s anti-inflammatory and vasculoprotective actions warrant future investigation.

In summary, we demonstrate for the first time that APN inhibits TNFα-induced inflammatory response via Cav1-mediated ceramidase recruitment and activation in an AdipoR1-dependent fashion. These experimental results not only deepen our understanding of APN biological signaling, but also suggest interventions facilitating AdipoR1/Cav1/nCDase signaling (such as upregulating AdipoR1 expression and stimulating nCDase) may be novel vasculoprotective targets in the diabetic patient.
SOURCES OF FUNDING
This research was supported by grants NIH HL-63828, HL-096686, American Diabetes Association 7-11-BS-93 (XLM), American Diabetes Association 1-11-JF56 (YJW), and American Diabetes Association 1-12-BS-180, NIH 5R01DK064344 (RS).

ACKNOWLEDGMENTS
We greatly appreciate Drs. Shey-Shing Sheu, Shi Pan in Department of Medicine and Gyorgy Hajnoczky in Department of Pathology, Anatomy, & Cell Biology for their instruction and assistance in performing the confocal microscopic analysis.

DISCLOSURES
None.

REFERENCES


FIGURE LEGENDS

**Figure 1.** Effect of AdipoR1/AdipoR2 knockdown upon APN’s anti-ICAM-1 effect. HUVEC were transfected with scramble or siRNA against AdipoR1/AdipoR2 to knockdown AdipoR expression (A). 48 hours after transfection, cells were pretreated with vehicle, gAPN or fAPN followed by TNFα treatment. Effect of APN upon TNFα-induced ICAM-1 expression (12 hours post-TNFα treatment) was determined in scramble (B), AdipoR1 siRNA (C) and AdipoR2 siRNA (D) transfected cells. *P<0.05, **P<0.01 vs. TNFα-treated animals without APN treatment.

**Figure 2.** Effect of AdipoR1/AdipoR2 knockdown upon APN’s anti-oxidative and anti-nitrative effect. Cells were treated as described in Figure 1. Effect of APN upon TNFα-induced superoxide production and nitrotyrosine formation was determined in scramble (A/D), AdipoR1 (B/E) and AdipoR2 (C/F) transfected cells. *P<0.05, **P<0.01 vs. TNFα-treated animals without APN treatment.

**Figure 3.** AdipoR1-dependent nCDase activation mediates APN anti-inflammatory action. (A): Transfection of HUVEC reduced nCDase expression (left panel) and blocked nCDase activation by APN (right panel); (B): Effect of AdipoR1 and AdipoR2 knockdown upon APN-induced nCDase activation; (C): Effect of nCDase knockdown upon the inhibitory action of APN upon TNFα-induced ICAM-1 expression. **P<0.01 vs. Con (A and B). *P<0.05, **P<0.01 vs. TNFα-treated animals without APN treatment (C).

**Figure 4.** Role of AMPK in APN-induced nCDase activation. Transfection of HUVEC with AMPKα siRNA reduced AMPKα expression (A) and blocked gAPN-induced ACC phosphorylation (B). In contrast, APN-induced nCDase activation was not affected (C) and APN’s anti-TNFα (ICAM-1 expression, D) was only partially inhibited when AMPK expression was genetically inhibited. *P<0.05, **P<0.01 vs. respective control (Con).

**Figure 5.** Role of Cav1 in APN-induced nCDase activation and anti-inflammatory action. HUVEC Cav1 expression was genetically inhibited by Cav1 siRNA (A, left panel). Cav1 knockdown blocked APN-induced nCDase activation (A, right panel) and anti-inflammatory effects of APN (ICAM-1 expression, B). However, Cav1-KD had no significant effect upon anti-inflammatory effects of S1P and SEW2187 (C). **P<0.01 vs. respective control (A) or vs. TNFα-treated animals without APN treatment (B/C).

**Figure 6.** Effect of AdipoR1/AdipoR2 or Cav1 knockdown upon cellular levels of ceramide (A) and S1P (B) after TNFα treatment in the presence and absence of gAPN. Representative photos from at least 5 repeated experiments. C: Cav1/AdipoR1 co-localization determined by immunofluorescent staining. Representative photos from at least 5 repeated experiments; D: Cav1/AdipoR1 interaction determined by co-immunoprecipitation in HUVEC. Cav1 was immunoprecipitated with antibody against Cav1 and samples were immunoblotted with antibody against AdipoR1 (upper panels). To immunoprecipitate AdipoR1, HUVEC were transfected with Myc-tagged AdipoR1 expressing vector and immunoprecipitated with antibody against Myc. Samples were then immunoblotted with antibody against Cav1 (lower panel). E: Cav1/AdipoR1 interaction determined by co-immunoprecipitation in RAEC. MUT=Re-expression in Cav1-KD cells of a mutated Cav1, in which 5 aromatic residues within the scaffolding domain responsible for Cav1 interaction with partner proteins were converted to alanine. F: Cav1/nCDase interaction determined by co-immunoprecipitation in aortic segment from mice treated with vehicle or gAPN. Representative blots from at least 5 repeated experiments; G: APN/Cav1 complex forming (upper panel) and lack of AdipoR2/Cav1 interaction determined by immunoprecipitation (lower panel). Representative blots from at least 5 repeated experiments.
Figure 7. Cellular distribution of nCDase without APN treatment (A: top panel) and effect of APN treatment upon Cav1 and nCDase interaction in WT, Cav1-KD or AdipoR1KD HUVEC (A: confocal microscopy; B: immunoprecipitation). Note that APN-enhanced Cav1/nCDase interaction is blocked by AdipoR1, but not AdipoR2 knockdown. Cav1/AdipoR1/nCDase complex formation determined by immunoprecipitation (C/D). Representative images from at least 5 repeated experiments/experimental conditions.

Figure 8. Effect of AdipoR1/AdipoR2 knockout upon APN inhibition of TNFα-induced leukocyte rolling and adhesion. WT, AdipoR1KO and AdipoR2KO mice were pre-treated with vehicle or gAPN and leukocyte rolling and adhesion was observed using intravital microscopy following TNFα injection. The inhibitory effect of APN upon TNFα-induced leukocyte rolling (A) and adhesion (B) observed in WT mice was virtually abolished in AdipoR1 but not in AdipoR2 knockout mice. **P<0.01 vs. TNFα-treated animals without APN treatment; †P<0.05, ‡P<0.01 vs. WT with the same treatment.
Novelty and Significance

What Is Known?

- Diabetes mellitus Type 2 diabetes affects over 20 million people in the United States and is a major risk factor for cardiovascular diseases.

- Adiponectin (APN), produced by adipocytes, protects against vascular injury from inflammation, through interaction with one or both of its receptor proteins (AdipoR1 or AdipoR2).

- APN has been shown to decrease inflammation by activating ceramidase (CDase). It is, however, unknown whether APN protects against vascular injury from inflammation by activating CDase or if AdipoR1/AdipoR2 themselves function as ceramidases.

What New Information Does This Article Contribute?

- APN decreases inflammation primarily through AdipoR1.

- The activation of CDase significantly contributes to APN’s anti-inflammatory function.

- The cell-signaling carrier protein caveolin 1 (Cav1) is essential for AdipoR1-mediated CDase activation, and the resultant anti-inflammatory actions of APN.

Patients with both Type 1 and Type 2 diabetes suffer more from thecardiovascular effects of inflammation than healthy individuals. Inflammation plays a major role in the cardiovascular complications of diabetes. Adiponectin is known to protect against cardiovascular injury caused by inflammation, through interaction with one or both of its receptor proteins (AdipoR1 and AdipoR2). It has been shown that APN decreases inflammation through the enzyme ceramidase; however, it remains unknown whether APN protects against vascular injury from inflammation by activating ceramidase or if AdipoR1/AdipoR2 themselves function as ceramidases. Here, we show that APN decreases inflammation primarily through AdipoR1. Ceramidase activation significantly contributes to the anti-inflammatory effects of APN. We demonstrate the necessity of Cav1 for AdipoR1-mediated ceramidase activation and the resultant anti-inflammatory actions of APN. Our work contributes to a better understanding of APN biological signaling. Furthermore, these findings provide the framework for new approaches (such as increasing AdipoR1 protein expression or stimulation ceramidase activity) to decrease vascular injury in patients with diabetes mellitus.
Figure 1
Figure 2
Figure 3

**A**

**B**

**C**
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Adiponectin Inhibits TNF-α-Induced Vascular Inflammatory Response via Caveolin-Mediated Ceramidase Recruitment and Activation
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Circ Res. published online January 7, 2014;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Adiponectin inhibits TNF-α-induced vascular inflammatory response via caveolin-mediated ceramidase recruitment and activation

Wang et al. APN in vascular inflammation

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SUPPLEMENTAL METHODS

Materials

Human umbilical vein endothelial cells (HUVEC), rat aortic endothelial cells (RAEC) and cell culture reagents were purchased from Cell Applications (San Diego, CA). Fetal bovine serum was from Hyclone (Logan, CT). Antibody against caveolin-1 (Cav1), Pan-cadherin and Cy5-conjugated secondary antibody were from Abcam. Antibodies against ICAM-1, ACC, phosphorylated ACC, and FITC, tetramethyl rhodamine (TRITC)-conjugated or horseradish peroxidase-conjugated secondary antibodies were from Cell Signaling Technology (Danvers, MA). Antibodies against AdipoR1 and AdipoR2 were from Bioss Inc (Woburn, MA). Antibodies against Ceramide and S1P were from Sigma (Saint Louis, MO) and Novus Biologicals (Littleton, CO). Recombinant human TNF-α protein (rhTNFα) and nCDase antibody were from R&D System (Minneapolis, MN). Recombinant human globular and full length APN (gAPN and fAPN) were from Peprotech, Inc (Rocky Hill, NJ). S1P and SEW2871 (a selective S1P receptor-1 agonist) were from Cayman Chemical (Ann Arbor, MI). S1P was dissolved in 70% ethanol. A stock solution for S1P was then made in 1% fatty acid–free bovine serum (FBS) albumin in PBS (137 mmol/L sodium chloride, 1.5 mmol/L potassium phosphate, 7.2 mmol/L sodium phosphate, 2.7 mmol/L potassium chloride, pH 7.4). An appropriate amount of the stock solution was then added to the cultured cells to yield the desired final concentration of S1P.

Cell Culture and Treatments

HUVEC (Passage 2-3) were plated on six-well plates and cultured in endothelial growth medium containing 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2. Upon 80% confluence, cells were treated with vehicle, gAPN (2 μg/ml) or fAPN (10 µg/ml). One hour after APN treatment, 10 ng/ml rhTNFα was added. Cells were collected 12 hours after TNFα treatment and oxidative/nitrative stress and ICAM-1 expression were determined as described in detail below.

Small Interfering RNA Transfection, Plasmid Construction, and Transfection

siRNA duplexes against Cav-1, AdipoR1, AdipoR2, AMPKα1, and nCDase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Universal control oligonucleotides (AllStars) from Santa Cruz served as negative control. HUVECs (80% confluent) were transfected via siIMPORTER siRNA transfection kit (Qiagen Science Inc. Benelux) per manufacturer’s protocol (final siRNA concentration: 50 nM). The cells were then incubated at 37°C with mixture of transfection regent and siRNA. After 5 hours, the cells in each well were replaced with fresh growth medium. At 72 hours after transfection, the cells transfected with control and experimental siRNA were used for experiments, harvested separately for extraction of total protein, and used for Western blot analysis or Immunoprecipitation assay. For plasmid construction, the cDNA encoding full-length (FL) human Cav-1 and muCav-1 were subcloned into pcDNA3.1 at Hind III and XbaI sites. The FL constructs then served as templates to further generate scaffolding domain alanine mutants (muCav-1) using standard PCR-based strategies. Scaffolding domain alanine region: DGIWKASFTFTVTKYWFYR; Alanine mutagenesis region:DGIWKASATTAAYTKYAYR. Endothelial cells were maintained in culture with 10% fetal bovine serum DMEM medium (Cell Applications, CA). For most experiments, cells in a
60-mm culture plate were transfected with 2μg of plasmid DNA encoding muCav-1, FLCav-1, using LipofectAMINE (Qiagen) according to the manufacturer’s protocols. When cells were co-transfected with plasmid DNA, empty vector (no cDNA insert) was used as control. Approximately 4 hours after transfection, culture medium was switched to 10% DMEM culture medium and incubation proceeded for 48 hours prior to the experiments. Then the cells will be harvested for extraction of total protein and used for the following experiments.

Ceramidase Enzyme Activity Assay

The nCDase enzyme activity was determined as previously reported, with minor modification26. Briefly, at experiment conclusion, cells were collected and washed twice with PBS. Cell pellets were resuspended in 100 μl 0.25 M sucrose solution, sonicated, and centrifuged at 15,000 g for 3 minutes. The supernatant was collected and protein concentration was determined. A 25 μl sample containing identical protein amount, 75μl 25 mM phosphate buffer (pH 7.4), and 0.5 μl 4 mM Rbm14-12 substrate solution in ethanol (final substrate concentration 40 μM; final ethanol concentration 1%) were loaded into each well of a 96-well plate. The same incubation mixture without supernatant served as negative control. The plate was incubated at 37°C for 1 hour without agitation. The enzymatic reaction was stopped by adding 25 μl methanol and 100 μl NaIO4 (2.5 mg/ml) in 200 mM glycine/NaOH buffer (pH 10.6) to each well. The plate was placed in a dark room for 1 hour. Fluorescent intensity was quantified via SpectraMax Microplate Reader (Molecular Devices, λex 355 nm, λem 446 nm).

Determination of Superoxide and Peroxynitrite Content

Superoxide content was quantified by lucigenin enhanced luminescence, and the cellular origin of reactive oxygen species was determined by dihydroethidium staining (DHE, Molecular Probes, Carlsbad, CA). Briefly, histological detection of superoxide anion in situ was performed using fresh-cultured endothelial cells stained with DHE (5 µmol/L) in medium for 5 minutes at 37°C. The intensity of the fluorescence signal was analyzed using IP lab Imaging Software 4.5 (BioVision, Rockingham, VT). Nitrotyrosine content, the footprint of peroxynitrite formation, was quantified by a modified ELISA procedure. In brief, endothelial cells were homogenized in ice cold PBS (1:10 w/v) using sonication with a dismembrator (Fisher Scientific, Pittsburgh, PA). The homogenates were centrifuged for 10 min at 12,000g at 4°C. The supernatants were collected and protein concentrations were determined by Bio-Rad method. A nitrated protein solution was prepared for use as a standard by adding 8 μl of chemically synthesized ONOO⁻ (concentration: 100-120 mM) to 3 ml of 0.04% (0.4 mg/ml) BSA in PBS. The amount of nitrotyrosine present in the peroxynitrite-treated BSA solution was measured at 430 nm using a spectrophotometer (Beckman DU 640, Fullerton, CA) and expressed as nanograms per milliliter. The stock solution of the peroxynitrite-treated BSA was diluted with PBS (final nitrotyrosine concentration, 0.75–75 ng/ml). These standard samples, along with samples from endothelial cells (protein concentration, 4 mg/ml) were applied to disposable sterile ELISA plates (Corning Glassworks, Corning, NY) and allowed to bind for 1 h at 37°C in a microincubator shaker (Teitec Co., San Jose, CA). After blocking nonspecific binding sites with 1% BSA in PBS, the wells were incubated for 60 min at 37°C with a rabbit polyclonal anti-nitrotyrosine primary antibody (Millipore) and subsequently for 60 min at 37°C with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000, Amersham Pharmacia Biotech, Inc. Piscataway, NJ). After washing the plates, the peroxidase reaction product was generated usingO-
phenylenediamine dihydrochloride (2.2 mM) (Abbott Diagnostics, Abbott Park, IL). The plate was incubated for 20 min in the dark at room temperature, and the reaction was stopped by addition of 20 ml of 2 M H2SO4. The optical density was measured at 460 nm with a SpectraMax L microplate reader (MD LLC, Sunnyvale, CA). The amount of nitrotyrosine content in samples was calculated using standard curves generated from nitrated BSA containing known amounts of nitrotyrosine.

Confocal Immunofluorescence Microscopy
30 minutes after vehicle or gAPN treatment, HUVEC were fixed with 4% paraformaldehyde/PBS in µ-Slide (ibidi LLC, Verona, WI) for 15 minutes followed by PBS washing. Cells were first treated with antibodies against AdipoR1, Cav1, or nCDase (at 1:200), followed by incubation with tetramethyl rhodamine (TRITC)-conjugated anti-rabbit IgG and Cy5-conjugated anti-goat IgG (1:200). For Ceramide and S1P staining, cells were fixed with 4% paraformaldehyde/PBA for 2 min. Cells were then washed with ddH2O (2 min) and TBS (pH 7.6, 5 min). Nonspecific binding sites were blocked by 2x casein solution (Vector Inc.) for 10 min at room temperature. The primary antibodies were prepared with TBS solution containing 5µM of CaCl2 and cells were incubated with antibody for 2 h at room temperature. After 10 min washing with TBS under agitation, cells were incubated with fluorescence labeled secondary antibody. After washing with PBS, coverslips were mounted utilizing an anti-fade solution (KPL, Gaithersburg, MD). Samples omitting the primary antibody served as negative control. Slides were visualized by a FV1000 confocal microscope with x60 oil-immersion objective lenses (Olympus, Tokyo, Japan). Fluorescent images were obtained by a digital camera and analyzed with Fluoview software (Olympus).

Immunoblotting and Co-Immunoprecipitation
HUVECs or RAECs were lysed with cold lysis buffer [50 mM Tris-HCl, pH 7.4/100 mM NaCl/0.1 mM EGTA/0.1 mM EDTA/1% Triton X-100/1 mM sodium orthovanadate/20 mM NaF/1 mM Na3P2O7 and cocktail protease inhibitor; For Cav-1 immunoprecipitation, 10mM Tris, pH 8.0/60 mM n-octyl β-D-glucopyranoside/150 mM NaCl/1 mM sodium orthovanadate/20 mM NaF/1 mM Na3P2O7 and cocktail protease inhibitor]29, 30. After homogenization and centrifugation, the supernatant was collected. For immunoblotting, proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were then incubated with primary antibodies (anti-ICAM-1, anti-nCDase, anti-ACC, anti-pACC, anti-AdipoR1, anti-Cav1, and anti-GAPDH) and HRP-conjugated secondary antibody. The blot was developed with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL). Bands were visualized by a Kodak Image Station 4000R Pro (Rochester, NY). For co-immunoprecipitation, cell lysates were pre-cleared with corresponding nonimmune IgG, and incubated together with protein A plus-Sepharose for 30 minutes at 4°C. Cleaned lysates were then incubated with 2 µg of either anti-Cav1 or anti-nCDase antibodies. Cell lysates were then incubated with protein A plus-Sepharose overnight at 4°C. Nonimmune rabbit IgG served as negative control. Protein A beads were then extensively washed with lysis buffer. Proteins were eluted from beads, and resolved by elusion buffer. Samples with 2XSDS sample buffer were heated and separated by electrophoresis. After transfer to PVDF membranes, proteins were immunoblotted with anti-AdipoR1 (for Cav1/AdipoR1 interaction) or anti-Cav1 (for Cav1/ceramidase interaction) as described above.
Intravital Microscopy Analysis of Leukocyte Rolling and Adhesion

Leukocyte rolling and adhesion was assayed in mesenteric post-capillary venules by intravital microscopy as we previously described\textsuperscript{31}. In brief, mice were pretreated with gAPN (first dose: 1.0 µg/g, i.p., 24 hours before TNF\(\alpha\) administration; second dose: 1 µg/g, i.p.). 30 min after the second dose of APN, mice were treated with recombinant TNF\(\alpha\) (1.0 µg/kg, i.p.) for 2 hours. Following exteriorization of a loop of ileum tissue via a midline laparotomy, the ileum was placed in a temperature-controlled fluid-filled Plexiglas chamber and trans-illuminated for bright-field observation of the peri-intestinal microcirculation. The ileum and mesentery were perfused throughout the experiment with a buffered K-H solution (pH 7.4, 37°C). Three to four straight, unbranched segments of post-capillary venules with lengths of >100 µm and diameters between 25 and 40 µm were studied in each mouse using an Eclipse FN1 Microscope (Nikon Corp), and the image recorded and analyzed on A WIN XP Imaging Workstation. Leukocyte rolling was defined as the number of leukocytes rolling past a fixed point per minute; leukocyte adherence was defined as the number of leukocytes firmly adhered to 100-µm length of endothelium for at least 30 seconds. Rolling and adhesion were quantitated 4 hours following TNF-\(\alpha\) injection. Venular blood velocity (V) was measured using the Microvessel Velocity OD-RT optical Doppler velocimeter (Circusoft Instrumentation) with corresponding software. Venular wall shear rate (\(\gamma\)) was calculated using the formula: \(\gamma=4.9\times8(V_{\text{mean}} /D)\), where D is the venule diameter.