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Adiponectin inhibits tumor necrosis factor- α -induced vascular inflammatory response via caveolin-mediated ceramidase recruitment and activation.

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Adiponectin Inhibits Tumor Necrosis Factor- α -Induced Vascular Inflammatory Response via Caveolin-Mediated Ceramidase Recruitment and Activation

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

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

Methods and Results: Adiponectin significantly reduced tumor necrosis factor (TNF) α -induced intercellular adhesion molecule-1 expression and attenuated TNF α -induced oxidative/nitrative stress in human umbilical vein endothelial cells. These anti-inflammatory actions were virtually abolished by adiponectin receptor 1 (AdipoR1-), but not AdipoR2-, knockdown (KD). Treatment with adiponectin significantly increased neutral ceramidase (nCDase) activity (3.7-fold; $P < 0.01$). AdipoR1-KD markedly reduced globular adiponectin-induced nCDase activation, whereas AdipoR2-KD only slightly reduced. More importantly, small interfering RNA-mediated nCDase-KD markedly blocked the effect of adiponectin on TNF α -induced intercellular adhesion molecule-1 expression. AMP-activated protein kinase-KD failed to block adiponectin-induced nCDase activation and modestly inhibited adiponectin anti-inflammatory effect. In contrast, in caveolin-1 KD (Cav1-KD) cells, >87% of adiponectin-induced nCDase activation was lost. Whereas adiponectin treatment failed to inhibit TNF α -induced intercellular adhesion molecule-1 expression, treatment with sphingosine-1-phosphate or SEW (sphingosine-1-phosphate receptor agonist) remained effective in Cav1-KD cells. AdipoR1 and Cav1 colocalized and coprecipitated in human umbilical vein endothelial cells. Adiponectin treatment did not affect this interaction. There is weak basal Cav1/nCDase interaction, which significantly increased after adiponectin treatment. Knockout of AdipoR1 or Cav1 abolished the inhibitory effect of adiponectin on leukocyte rolling and adhesion in vivo.

Conclusions: These results demonstrate for the first time that adiponectin inhibits TNF α -induced inflammatory response via Cav1-mediated ceramidase recruitment and activation in an AdipoR1-dependent fashion. (*Circ Res.* 2014;114:792-805.)

Key Words: adipokines ■ endothelial cells ■ inflammation ■ sphingolipids ■ vascular system injuries

Cardiovascular complications are the leading cause of death for patients with type 2 diabetes mellitus, a disease affecting >20 million people in the United States.¹ The inflammatory response associated with diabetes mellitus and the resultant vascular injury initiate more severe diabetic cardiovascular complications, including atherosclerosis and ischemic heart disease.² Defining the mechanisms leading to inflammatory vascular injury in diabetes mellitus and

identifying novel therapeutic strategies capable of protecting vascular function are therefore in great need.

Adiponectin is an adipocyte-derived cytokine. In contrast to the majority of adipokines (eg, tumor necrosis factor [TNF]- α), which are proinflammatory and significantly increased in patients with diabetes mellitus, adiponectin is a potent vascular protective molecule that is markedly reduced in patients with type 2 diabetes mellitus.³⁻⁵ Adiponectin

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Nonstandard Abbreviations and Acronyms

AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
AMPK	AMP-activated protein kinase
Cav1	caveolin-1
fAPN	full-length adiponectin
gAPN	globular adiponectin
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
KD	knockdown
KO	knockout
nCDase	neutral ceramidase
siRNA	small interfering RNA
TNF	tumor necrosis factor

reduces oxidative/nitrative stress, protects endothelial cells from apoptosis, inhibits leukocyte–endothelial interaction, and decreases smooth muscle proliferation.⁶ Two adiponectin receptors (AdipoR1 and AdipoR2) have been cloned.⁷ They belong to a new family of membrane receptors (the progestin and adiponectin, C1Q and collagen domain-containing receptor superfamily)^{8–10} predicted to contain 7 transmembrane domains but are topologically distinct from G protein coupled receptor. Although both AdipoR1 and AdipoR2 are expressed in vascular endothelial cells, their roles in adiponectin-mediated anti-inflammatory and vascular protective actions have not been clarified.

Vasodilatory and vascular protective effects of adiponectin have been attributed previously to AMP-activated protein kinase (AMPK)–mediated endothelial nitric oxide synthase phosphorylation and nitric oxide production.^{11,12} However, we recently demonstrated that in the ischemic/reperfused heart, adiponectin's antioxidative/antinitrative effects are largely AMPK independent.^{13,14} Moreover, a recent study demonstrates that adiponectin activates neutral ceramidase (nCDase) in an adiponectin receptor–dependent but AMPK-independent fashion.¹⁵ As ceramidase is a key enzyme converting ceramide, a proinflammatory molecule, to sphingosine-1-phosphate (S1P), an anti-inflammatory and cardiovascular protective molecule,^{16–20} it is possible that adiponectin'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 adiponectin receptor subtype responsible for vascular protective action; (2) determine the role of ceramidase activation in adiponectin-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

Human umbilical vein endothelial cells (HUVECs; passage 2–3) were plated on 6-well plates and cultured in endothelial growth medium containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO₂.

On 80% confluence, cells were treated with vehicle, globular adiponectin (gAPN; 2 µg/mL)¹⁴ or full-length adiponectin (fAPN; 10 µg/mL). One hour after adiponectin treatment, 10 ng/mL recombinant human TNFα protein²¹ was added. Cells were collected 12 hours after TNFα treatment and oxidative/nitrative stress and intercellular adhesion molecule-1 (ICAM-1) expression were determined as described in detail below.

Small Interfering RNA Transfection, Plasmid Construction, and Transfection

Small interfering RNA (siRNA) duplexes against Cav1,²² AdipoR1,²³ AdipoR2,²³ AMPKα1,²³ 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 nmol/L).

Ceramidase Enzyme Activity Assay

The nCDase enzyme activity was determined as previously reported, with minor modification.²⁵ Briefly, at experiment conclusion, cells were collected and washed twice with PBS. Cell pellets were resuspended in 100-µL 0.25-mol/L sucrose solution, sonicated, and centrifuged at 15 000g for 3 minutes. The supernatant was collected and protein concentration was determined. A 25-µL sample containing identical protein amount, 75-µL 25-mmol/L phosphate buffer (pH 7.4), and 0.5-µL 4-mmol/L Rbm14-12 substrate solution in ethanol (final substrate concentration 40 µmol/L; 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-mmol/L 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, lex 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 (Molecular Probes, Carlsbad, CA) as previously described.²⁶ Nitrotyrosine content, the footprint of peroxynitrite formation, was quantified by ELISA as previously described.^{26,27}

Confocal Immunofluorescence Microscopy

Thirty minutes after vehicle or gAPN treatment, HUVECs 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, caveolin-1 (Cav1), or nCDase, followed by incubation with tetramethyl rhodamine–conjugated antirabbit IgG and Cy5-conjugated anti-goat IgG. For ceramide and S1P staining, cells were fixed with 4% paraformaldehyde/PBA for 2 minutes. Cells were then washed with ddH₂O (2 minutes) and TBS (pH 7.6; 5 minutes). The primary antibodies were prepared with TBS solution containing 5 µmol/L of CaCl₂ and cells were incubated with antibody for 2 hours at room temperature. After 10-minute washing with TBS under agitation, cells were incubated with fluorescence-labeled secondary antibody. Slides were visualized by a FV1000 confocal microscope with ×60 oil-immersion objective lenses (Olympus, Tokyo, Japan). Fluorescent images were obtained by a digital camera and analyzed with Fluoview software (Olympus).

Immunoblotting and Coimmunoprecipitation

HUVECs were lysed with cold lysis buffer.^{28,29} 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 horseradish peroxidase-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 coimmunoprecipitation, cell lysates were precleared 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 washed extensively with lysis buffer. Proteins were eluted from beads and resolved by elution buffer. Samples with 2×SDS sample buffer were heated and separated by electrophoresis. After transfer to polyvinylidene fluoride 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 were assayed in mesenteric post-capillary venules by intravital microscopy as we previously described.³⁰ In brief, mice were pretreated with gAPN (first dose: 1.0 µg/g, IP, 24 hours before TNFα administration; second dose: 1 µg/g, IP). Thirty minutes after the second dose of adiponectin, mice were treated with recombinant TNFα (1.0 µg/kg, IP) for 2 hours. After exteriorization of a loop of ileum tissue via a mid-line laparotomy, the ileum was placed in a temperature-controlled fluid-filled Plexiglas chamber and transilluminated 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 4 straight, unbranched segments of postcapillary 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 was 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 ≥30 seconds. Rolling and adhesion were measured 4 hours after TNFα 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 ($\dot{\gamma}$) was calculated using the formula: $\dot{\gamma}=4.9\times 8(V_{\text{mean}}/D)$, where *D* is the venule diameter.

Statistical Analysis

All values in the text and figures are presented as mean±SEM of *n* independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Tukey correction for post hoc *t* test. Western blot densities were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test. Probabilities of ≤0.05 were considered statistically significant.

Results

Anti-Inflammatory, Antioxidative, and Antinitrative Effects of Adiponectin Are Mediated Largely by AdipoR1

Treatment of HUVEC with TNFα elicits significant inflammatory response³¹ as evidenced by upregulated 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*>0.1 versus vehicle-treated group). AdipoR1-knockdown (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 versus 3.36±0.48 pmol/mg protein; *P*<0.05). AdipoR2-KD did not significantly modify the TNFα-induced inflammatory reaction without adiponectin treatment (Figure 1D). Most importantly, in AdipoR1-KD HUVEC, the inhibitory effects of gAPN on 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), antioxidative (Figure 2C), and antinitrative effects (Figure 2F) of gAPN were largely retained. In a separate experiment, the influence of AdipoR1-KD and AdipoR2-KD on the anti-inflammatory effects of 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 blocked largely by AdipoR1-KD (>76% inhibition; *P*>0.05 versus TNFα with vehicle; Figure 1C) and blocked modestly by AdipoR2-KD (<31% inhibition; *P*<0.05 versus TNFα with vehicle; Figure 1D). These results demonstrate that AdipoR1 largely mediates the anti-inflammatory effect of adiponectin in HUVEC.

Activation of nCDase by gAPN Contributes to Its Anti-Inflammatory Action

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

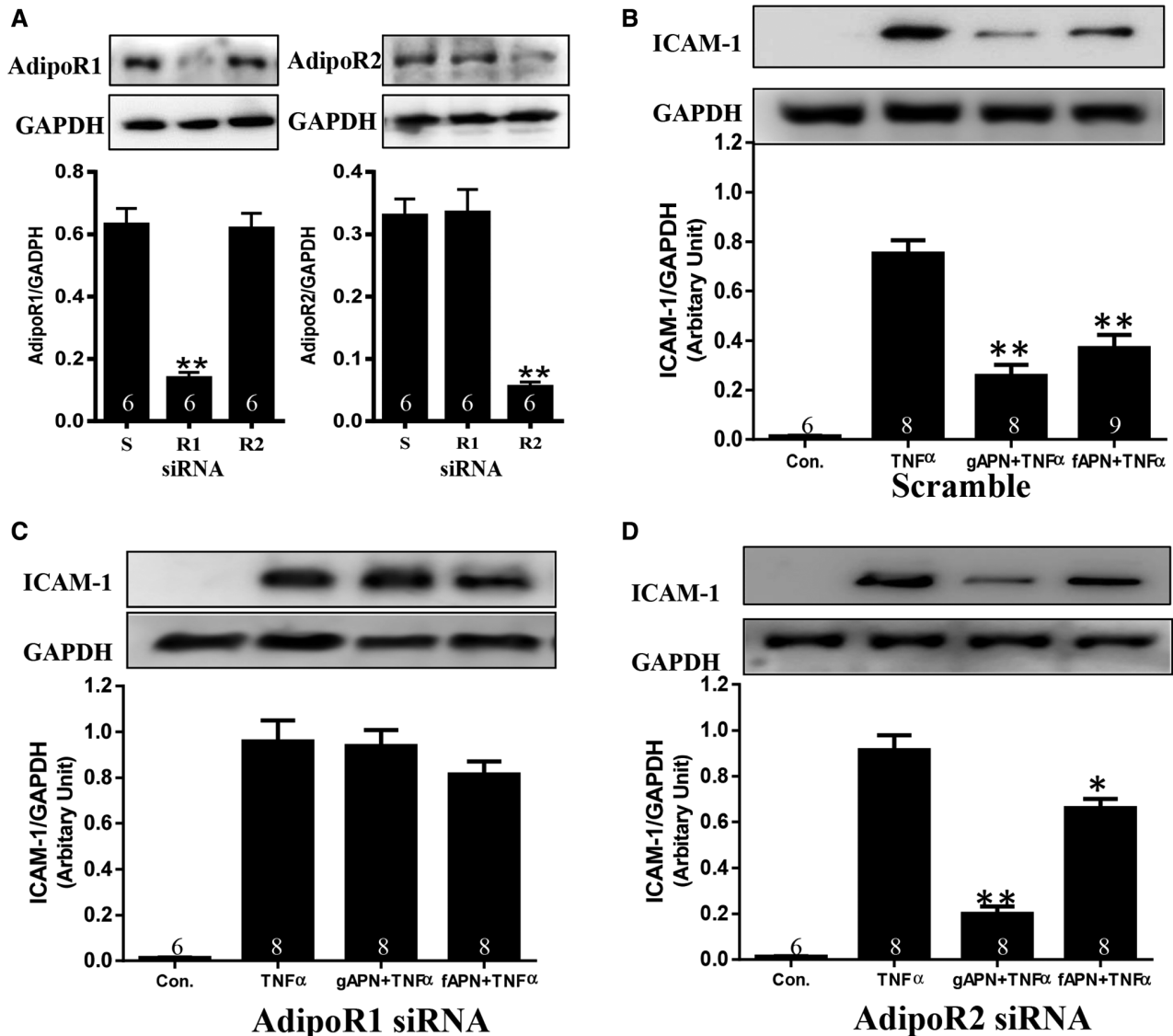


Figure 1. Effect of adiponectin receptor 1 (AdipoR1)/AdipoR2 knockdown on adiponectin (APN)'s anti-intercellular adhesion molecule-1 (ICAM-1) effect. Human umbilical vein endothelial cells (HUVECs) were transfected with scramble or small interfering RNA (siRNA) against AdipoR1/AdipoR2 to knockdown AdipoR expression (A). Forty-eight hours after transfection, cells were pretreated with vehicle, globular adiponectin (gAPN) or full-length adiponectin (fAPN) followed by tumor necrosis factor (TNF) α treatment. Effect of APN on TNF α -induced ICAM-1 expression (12 hours post-TNF α treatment) was determined in scramble (B), AdipoR1 siRNA (C), and AdipoR2 siRNA (D) transfected cells. * P <0.05, ** P <0.01 vs TNF α -treated animals without APN treatment.

Role of AMPK in Adiponectin Activation of nCDase and Anti-Inflammatory Action

AMPK is a well-recognized downstream signaling molecule of adiponectin.³² To determine the relationship between AMPK and nCDase in relation to adiponectin's anti-inflammatory action, AMPK expression was inhibited genetically by siRNA (Figure 4A). As summarized in Figure 4B, transfection with siRNA against AMPK α successfully blocked AMPK-mediated acetyl-CoA carboxylase phosphorylation after adiponectin treatment. However, AMPK-KD had no effect on gAPN-induced nCDase activation (Figure 4C). Finally, AMPK-KD partially blocked the effect of gAPN on TNF α -induced ICAM-1 expression (Figure 4D). These results demonstrate that adiponectin activates nCDase in an AMPK-independent manner, but its anti-inflammatory effect is partially mediated by AMPK signaling.

Role of Cav1 in Adiponectin-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 adiponectin signalsome, Cav3 (the primary caveolin isotype expressed in cardiomyocytes) plays an essential role in adiponectin's cardioprotective actions post myocardial ischemia/reperfusion.³³ To clarify whether Cav1 (the primary caveolin isotype expressed in endothelial cells)^{34,35} is required for adiponectin activation of nCDase and anti-inflammatory action in HUVEC, Cav1 expression was downregulated by siRNA (Figure 5A, left). As summarized in Figure 5, Cav1-KD not only blocked adiponectin activation of nCDase activity (Figure 5A, right) but also virtually abolished the inhibitory effect of adiponectin on TNF α -induced ICAM-1 expression (Figure 5B). To obtain more evidence

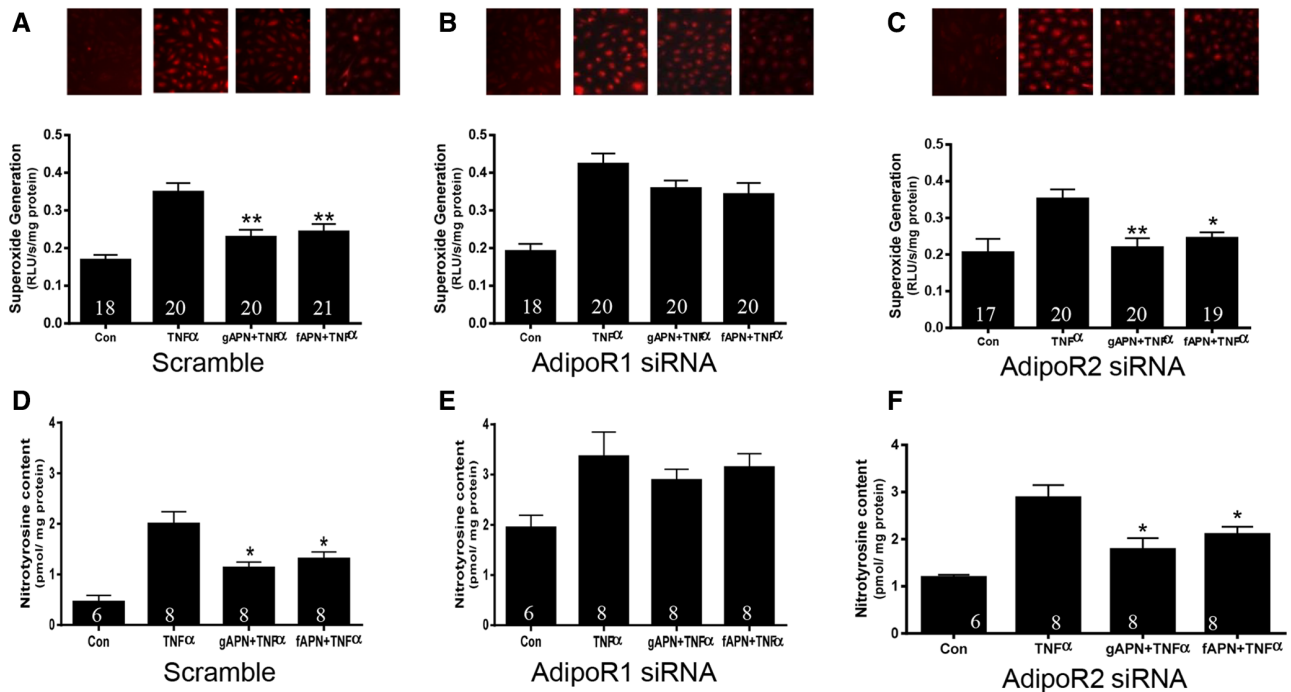


Figure 2. Effect of adiponectin receptor 1 (AdipoR1)/AdipoR2 knockdown on adiponectin (APN)'s antioxidant and antinitrative effect. Cells were treated as described in Figure 1. Effect of APN on tumor necrosis factor (TNF) α -induced superoxide production and nitrotyrosine formation was determined in scramble (A and D), AdipoR1 (B and E), and AdipoR2 (C and F) transfected cells. * P <0.05, ** P <0.01 vs TNF α -treated animals without APN treatment. fAPN indicates full-length adiponectin; and gAPN, globular adiponectin.

that adiponectin activates nCDase and inhibits inflammatory response in a Cav1-dependent manner, the effect of S1P and SEW2871 (a selective S1P receptor 1 agonist) on TNF α -induced ICAM-1 expression was compared in wild-type (WT) and Cav1-KD cells. As summarized in Figure 5C, treatment with either S1P or SEW2871 significantly inhibited TNF α -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 TNF α -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 and nCDase, Forming a Signaling Complex

Results presented in Figures 3B and 5A demonstrated that adiponectin increases nCDase activity in an AdipoR1 and Cav1-dependent fashion. To determine whether the effect of adiponectin on cellular levels of ceramide and S1P is also AdipoR1/Cav1 dependent, additional experiment was performed. As illustrated in Figure 6A and 6B, treatment with adiponectin significantly attenuated TNF α -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.³⁶ In a final attempt to clarify how Cav1 enables gAPN-initiated nCDase activation, 2 series of experiments were performed. First, the interaction between Cav1 and AdipoR1 was determined by immunofluorescent microscopy and coimmunoprecipitation. As illustrated in Figure 6C, colocalization of Cav1 and AdipoR1 was clearly observed (top). Cav1-KD did not alter AdipoR1 membrane localization (Figure 6C, bottom). Cav1 and AdipoR1 were coimmunoprecipitated in HUVEC (Figure 6D) and rat aortic endothelial cells (Figure 6E). Treatment with adiponectin had no significant effect on this protein-protein interaction (Figure 6D and 6E). Moreover, re-expression of WT Cav1 in Cav1-KD cells restored Cav1/AdipoR1 interaction (Figure 6D and 6E, right, 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,³⁷ did not reestablish Cav1/AdipoR1 interaction (Figure 6D and 6E, right, second lane). Moreover, Cav1/nCDase interaction was also observed in aortic segment from mice treated with vehicle or gAPN (Figure 6F). Finally, coimmunoprecipitation identified adiponectin /Cav1 interaction, indicating that adiponectin binds the proposed Cav1/AdipoR1 complex (Figure 6G, upper). These results support basal Cav1/AdipoR1 interaction, a protein-protein interaction requisite for adiponectin activation of nCDase.

In a separate experiment, potential Cav1/AdipoR2 interaction was determined by coimmunoprecipitation. As illustrated in Figure 6G (lower), interaction between Cav1/AdipoR2 is

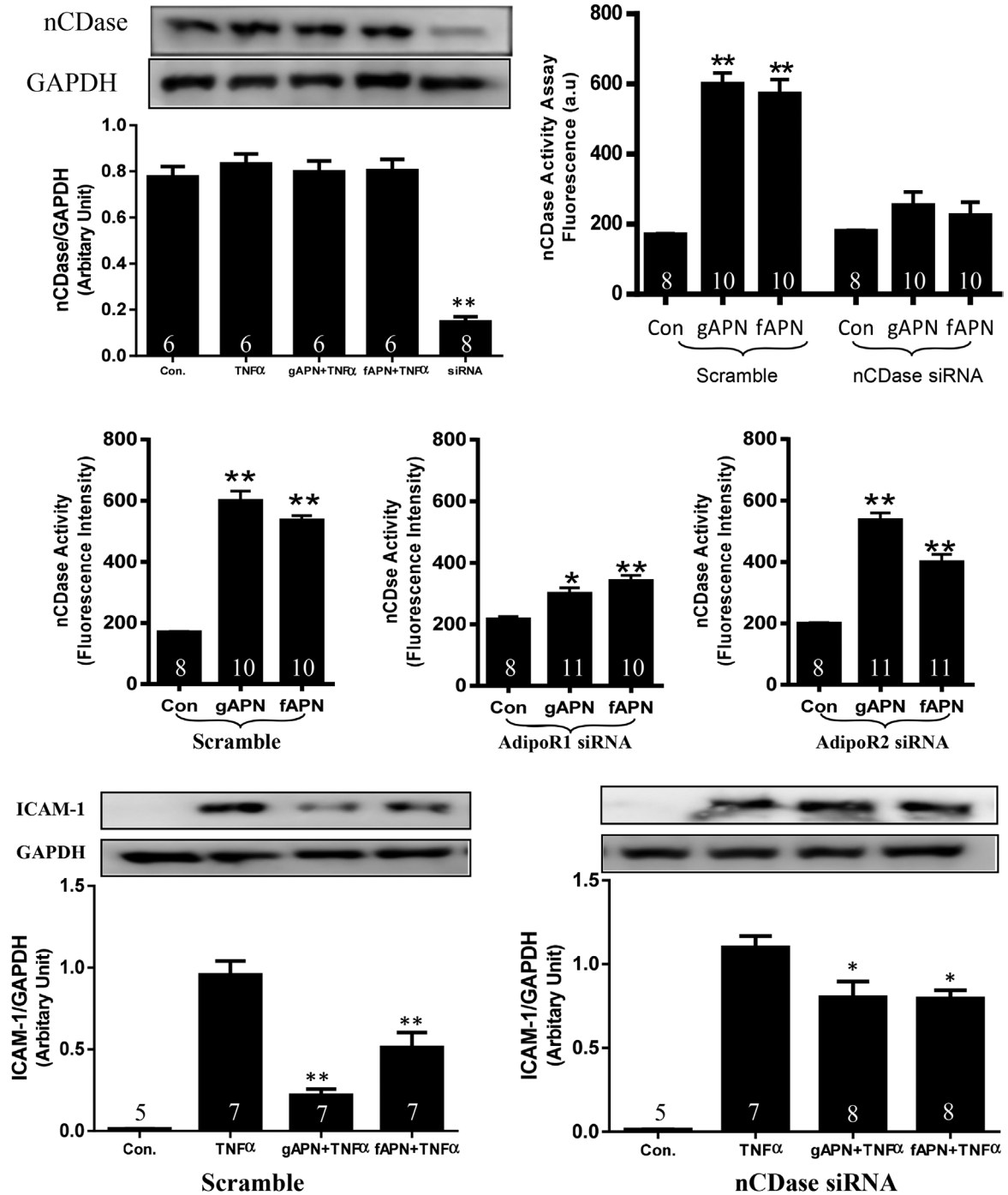


Figure 3. Adiponectin receptor 1 (AdipoR1)-dependent neutral ceramidase (nCDase) activation mediates adiponectin (APN) anti-inflammatory action. **A**, Transfection of human umbilical vein endothelial cells reduced nCDase expression (left) and blocked nCDase activation by APN (right). **B**, Effect of AdipoR1 and AdipoR2 knockdown on APN-induced nCDase activation. **C**, Effect of nCDase knockdown on the inhibitory action of APN on tumor necrosis factor (TNF) α -induced intercellular adhesion molecule-1 (ICAM-1) expression. ** $P < 0.01$ vs Con (**A** and **B**). * $P < 0.05$, ** $P < 0.01$ vs TNF α -treated animals without APN treatment (**C**). fAPN indicates full-length adiponectin; and gAPN, globular adiponectin.

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 2 caveolin binding motifs, of which one is located within its cytosolic region, whereas AdipoR2 contains only 1 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 colocalization of Cav1 and nCDase is not robustly apparent (Figure 7A), and coimmunoprecipitation of Cav1 and nCDase is weak (Figure 7A). However, gAPN treatment significantly increased Cav1/nCDase interaction, as evidenced by clear colocalization (Figure 7A) and strong

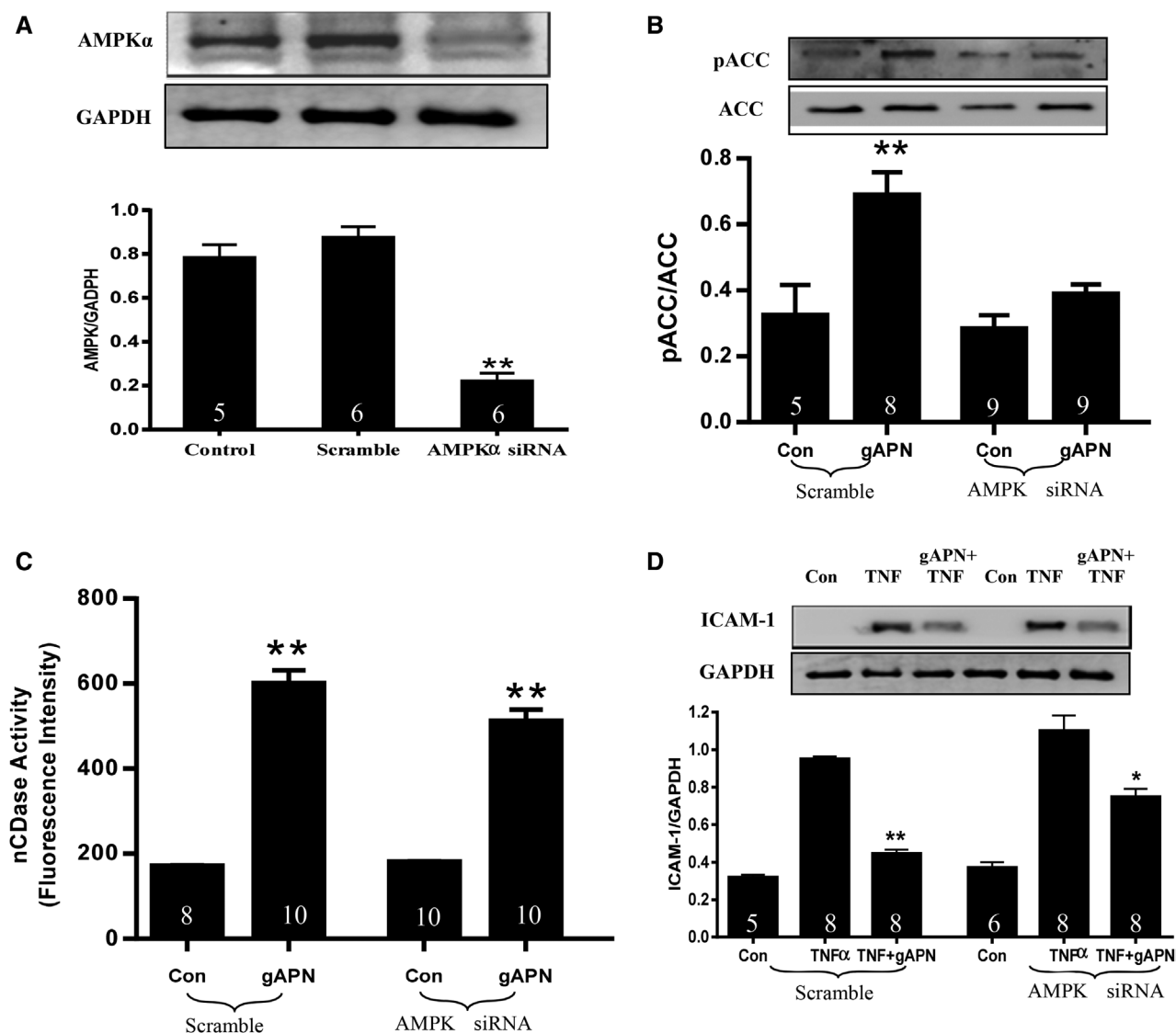


Figure 4. Role of AMP-activated protein kinase (AMPK) in adiponectin (APN)-induced neutral ceramidase (nCDase) activation. Transfection of human umbilical vein endothelial cells with AMPK α small interfering RNA (siRNA) reduced AMPK α expression (A) and blocked globular adiponectin (gAPN)-induced acetyl-CoA carboxylase (ACC) phosphorylation (pACC; B). In contrast, APN-induced nCDase activation was not affected (C) and APN's anti-tumor necrosis factor (TNF) α (intercellular adhesion molecule-1 [ICAM-1] expression, D) was only partially inhibited when AMPK expression was genetically inhibited. * $P < 0.05$, ** $P < 0.01$ vs respective control (Con).

coimmunoprecipitation (Figure 7B and 7C). Although the weak basal Cav1/nCDase interaction is not affected by AdipoR1-KD, the adiponectin-stimulated enhancement of Cav1/nCDase interaction is inhibited in AdipoR1-KD cells, but not altered in AdipoR2-KD 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 adiponectin. In contrast, basal Cav1/nCDase interaction is weak and is markedly enhanced after adiponectin treatment. These results suggest that AdipoR1–Cav1–nCDase forms a complex and their association is enhanced after adiponectin treatment. To obtain more evidence supporting this notion, AdipoR1/nCDase interaction in the presence and absence of adiponectin was determined. As illustrated in Figure 7D, weak AdipoR1/nCDase interaction was

observed and treatment with adiponectin significantly enhanced interaction.

Inhibitory Effect of Adiponectin on 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-KO on adiponectin inhibition of TNF α -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,³⁰ in vivo treatment of WT mice with gAPN significantly inhibited TNF α -induced leukocyte rolling and adhesion (Figure 8). Cav1-KO only slightly

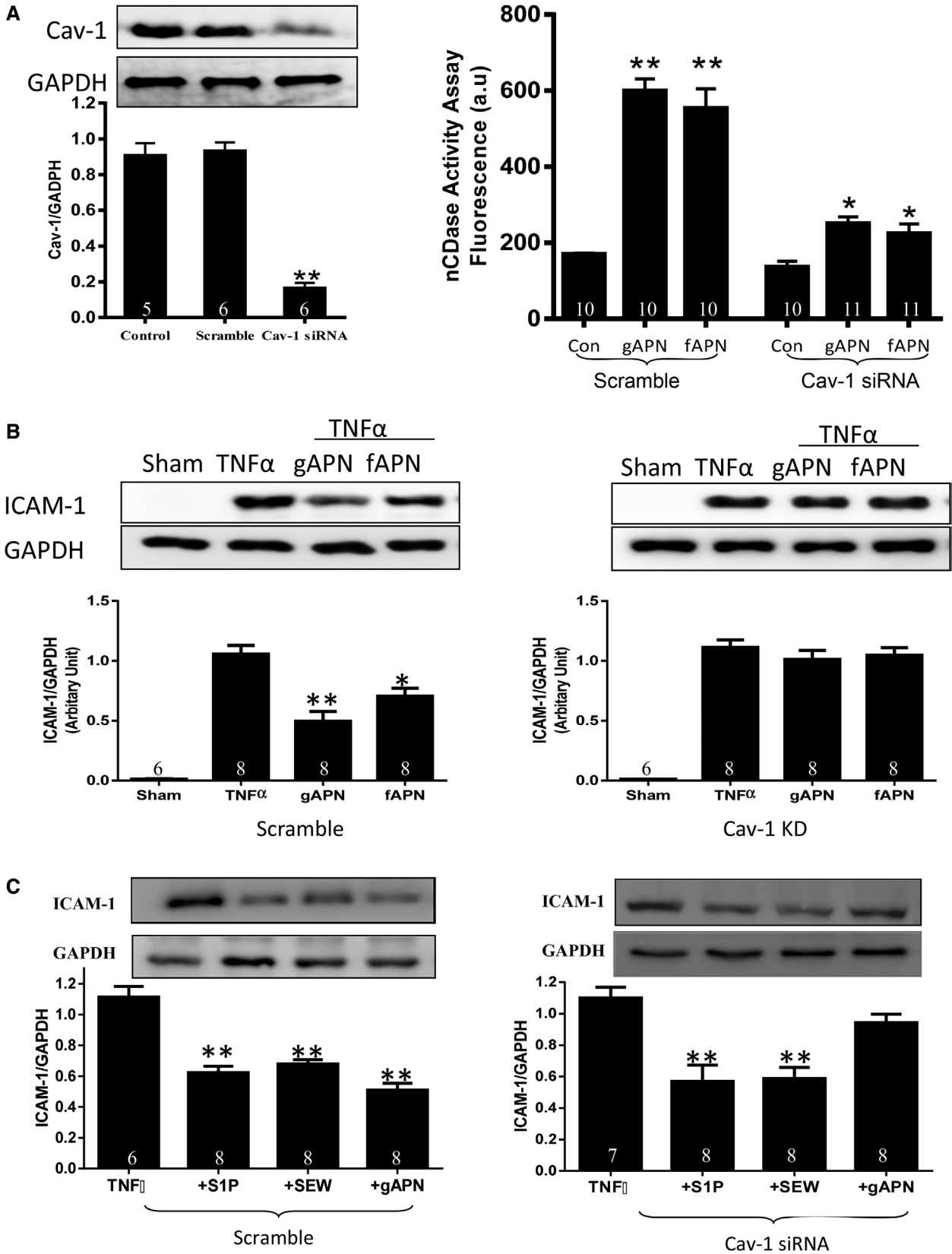


Figure 5. Role of caveolin-1 (Cav1) in adiponectin (APN)-induced neutral ceramidase (nCDase) activation and anti-inflammatory action. Human umbilical vein endothelial cell Cav1 expression was genetically inhibited by Cav1 small interfering RNA (siRNA; **A, left**). Cav1 knockdown (KD) blocked APN-induced nCDase activation (**A, right**) and anti-inflammatory effects of APN (intercellular adhesion molecule-1 [ICAM-1] expression, **B**). However, Cav1-KD had no significant effect on anti-inflammatory effects of sphingosine-1-phosphate (S1P) and a selective S1P receptor 1 agonist (SEW2871; **C**). ** $P < 0.01$ vs respective control (**A**) or vs tumor necrosis factor (TNF) α -treated animals without APN treatment (**B** and **C**). fAPN indicates full-length adiponectin; and gAPN, globular adiponectin.

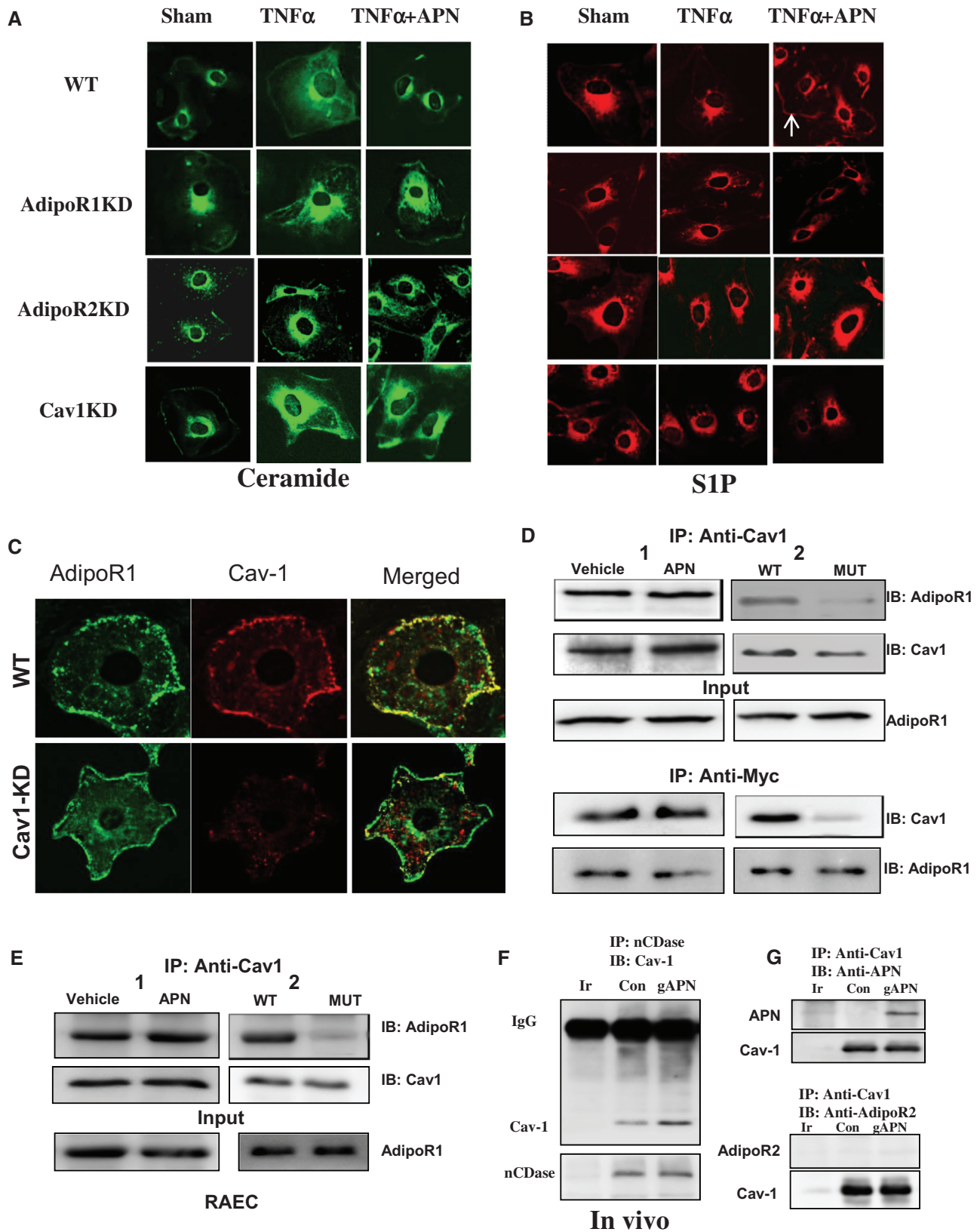


Figure 6. Effect of adiponectin receptor 1 (AdipoR1)/AdipoR2 or caveolin-1 (Cav1) knockdown (KD) on cellular levels of ceramide (A) and sphingosine-1-phosphate (S1P; B) after tumor necrosis factor (TNF) α treatment in the presence and absence of globular adiponectin (gAPN). Representative photos from ≥ 5 repeated experiments. C, Cav1/AdipoR1 colocalization determined by immunofluorescent staining. Representative photos from ≥ 5 repeated experiments. D, Cav1/AdipoR1 interaction determined by coimmunoprecipitation in human umbilical vein endothelial cells (HUVECs). Cav1 was immunoprecipitated with antibody against Cav1 and samples were immunoblotted with antibody against AdipoR1 (upper). To immunoprecipitate AdipoR1, HUVECs were transfected

Figure 6 (Continued). with Myc-tagged AdipoR1 expressing vector and immunoprecipitated with antibody against Myc. Samples were then immunoblotted with antibody against Cav1 (**lower**). **E**, Cav1/AdipoR1 interaction determined by coimmunoprecipitation in rat aortic endothelial cells (RAECs). Re-expression in Cav1-KD cells of a mutated Cav1 (MUT), 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 coimmunoprecipitation in aortic segment from mice treated with vehicle or gAPN. Representative blots from ≥ 5 repeated experiments. **G**, APN/Cav1 complex forming (**upper**) and lack of AdipoR2/Cav1 interaction determined by immunoprecipitation (**lower**). Representative blots from ≥ 5 repeated experiments. IB indicates immunoblotting; WT, wild-type.

($P > 0.05$) increased leukocyte rolling and adhesion after TNF α treatment, likely because of the increased basal nitric oxide production in these animals. In AdipoR1-KO mice, greater leukocyte rolling and adhesion were observed after TNF α treatment ($P < 0.05$ versus WT). Most importantly, the inhibitory effect of adiponectin on TNF α -induced leukocyte rolling and adhesion observed in WT mice was markedly inhibited in AdipoR1-KO mice (Figure 8) and completely abolished in Cav1-KO mice (leukocyte rolling, 45 ± 3.3 cells/100 μm with adiponectin treatment versus 48 ± 3.8 cells/100 μm with vehicle, $P > 0.1$; leukocyte adhesion, 6.4 ± 0.4 cells/100 μm with adiponectin versus 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 adiponectin on TNF α -induced leukocyte rolling and adhesion was largely preserved in AdipoR2-KO mice (Figure 8). Leukocyte rolling

was slightly decreased in AdipoR2-KO mice compared with AdipoR1-KO mice during basal conditions (before TNF α challenge), without statistically significant difference. This result is consistent with previous observations that no significant phenotype manifests in adiponectin-KO mice, unless challenged by metabolic or inflammatory stress.

Discussion

We have made 3 novel observations in this study. First, we demonstrate that the anti-inflammatory (determined by ICAM-1 expression), antioxidative (determined by superoxide production), and antinitrative (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 demonstrated previously that AdipoR1 has greater

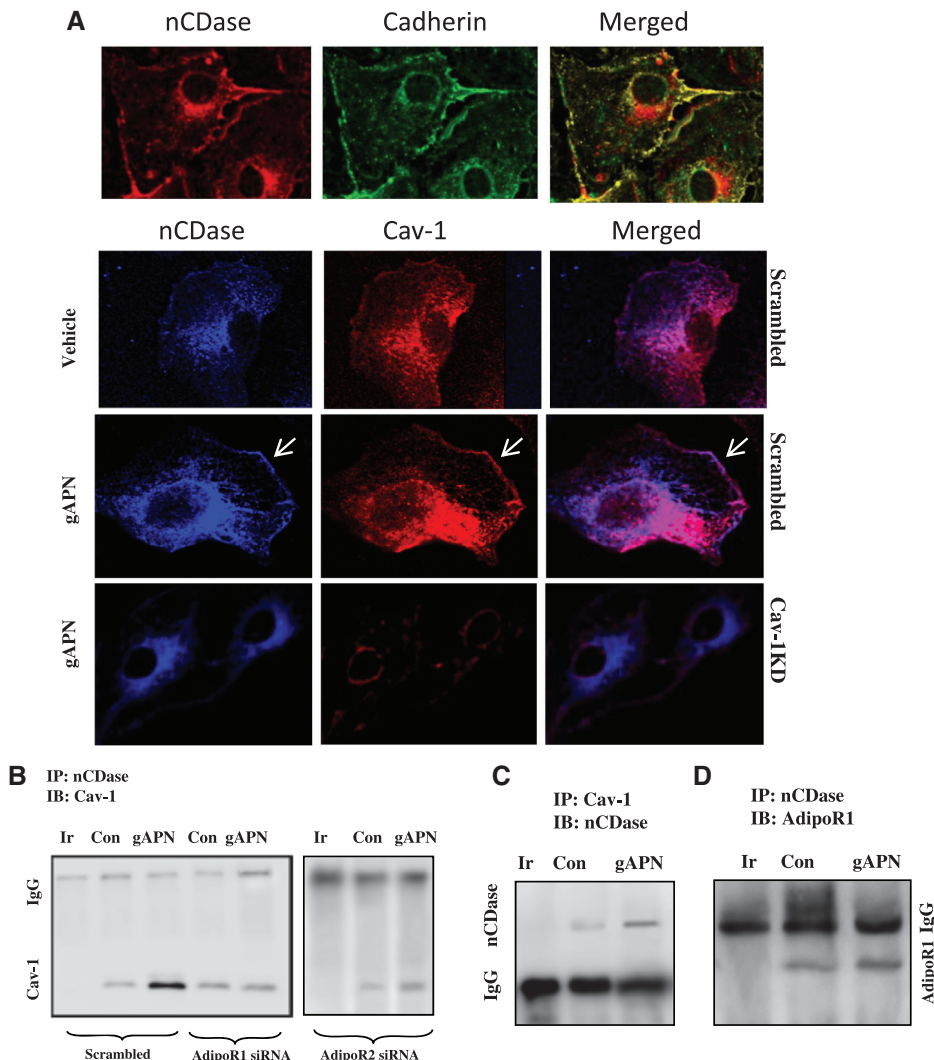


Figure 7. Cellular distribution of neutral ceramidase (nCDase) without adiponectin (APN) treatment (A, top) and effect of APN treatment on caveolin-1 (Cav1) and nCDase interaction in wild-type (WT), Cav1-KD, or AdipoR1-KD human umbilical vein endothelial cells (HUVECs; A, confocal microscopy; B, immunoprecipitation). Note that APN-enhanced Cav1/nCDase interaction is blocked by AdipoR1, but not AdipoR2 knockdown. Cav1/AdipoR1/nCDase complex formation determined by immunoprecipitation (C and D). Representative images from ≥ 5 repeated experiments/experimental conditions. gAPN indicates globular adiponectin.

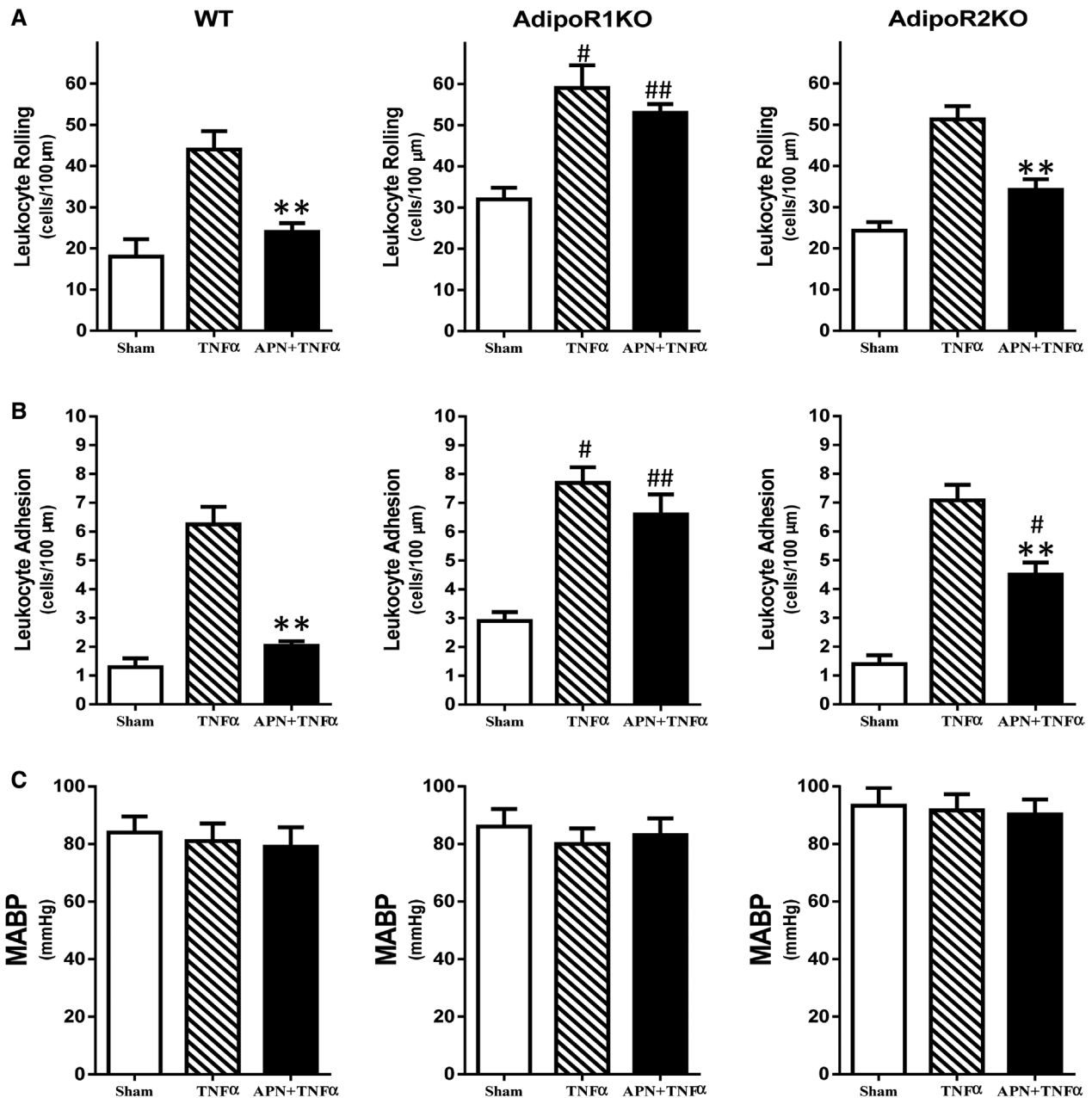


Figure 8. Effect of adiponectin receptor 1 (AdipoR1)/AdipoR2 knockout (KO) on adiponectin (APN) inhibition of tumor necrosis factor (TNF) α -induced leukocyte rolling and adhesion. Wild-type (WT), AdipoR1-KO, and AdipoR2-KO mice were pretreated with vehicle or globular adiponectin and leukocyte rolling and adhesion were observed using intravital microscopy after TNF α injection. The inhibitory effect of APN on TNF α -induced leukocyte rolling (A) and adhesion (B) observed in WT mice was virtually abolished in AdipoR1 but not in AdipoR2-KO mice. No significant difference was observed in mean arterial blood pressure (MABP) among different treatment group (C). ** $P < 0.01$ vs TNF α -treated animals without APN treatment; # $P < 0.05$, ## $P < 0.01$ vs WT with the same treatment.

gAPN binding affinity than AdipoR2.^{38,39} However, our results indicating fAPN (which has comparable binding affinity to both AdipoR1 and AdipoR2) exhibits an anti-inflammatory effect with stronger dependency on AdipoR1 argues against this possibility. A more likely explanation concerns prevalence. As AdipoR1 is the dominant adiponectin receptor isotype expressed in vascular tissue,³⁹ it stands to reason AdipoR1 would be chiefly responsible for the biological effects of adiponectin within the vascular system. Another possibility involves isotype-specific intracellular signaling. It is well recognized that membrane receptors possessing multiple subtypes (such

as TNF α 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, antioxidative, and antinflammatory 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 adiponectin) 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 adiponectin production and resultant cardiac protection as a result of rosiglitazone treatment are primarily mediated by AdipoR1 activation.⁴⁰

Second, we demonstrate that gAPN activates HUVEC nCDase in an AMPK-independent fashion and nCDase activation significantly contributes to adiponectin's anti-inflammatory function. In recent years, the biological activities of adiponectin have been investigated extensively. Four major functions (including metabolism-regulatory, anti-inflammatory, vasculoprotective, and cardioprotective effects) have been identified.⁴¹ Among multiple intracellular molecules activated after adiponectin 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 adiponectin biological functions.³² However, accumulating evidence suggests that the degree of AMPK involvement in biological regulation of adiponectin is dependent on organ and disease.⁴² Specifically, pharmacological inhibition of AMPK activity or genetic inhibition of AMPK expression virtually abolishes the central metabolic actions of adiponectin,^{43,44} indicating that AMPK plays an essential role in hepatic metabolic regulation of adiponectin. However, we¹³ and others⁴⁵ have demonstrated recently that the anti-ischemic/cardioprotective effects of adiponectin are largely AMPK-independent and involve the cyclooxygenase-2 and nuclear factor κ B-mediated signaling pathways. Moreover, a recent elegant study reported that adiponectin exerts antiapoptotic and cellular protective actions in an AMPK-independent, nCDase-dependent manner.¹⁵ Our current study demonstrates that unlike adiponectin's metabolic and antiapoptotic actions (largely AMPK-dependent or largely AMPK-independent), adiponectin's anti-inflammatory function is mediated by both the traditional AMPK pathway and the newly identified AMPK-independent nCDase activation pathway.

Third, 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 adiponectin. At least 2 possibilities exist that may explain the necessity of Cav1 in adiponectin vascular signaling. First, as suggested by a recent experimental study, AdipoR1 may exert nCDase activity and catalyze the ceramide reaction. Because many cofactors 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 adiponectin 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 coimmunoprecipitates with AdipoR1, suggesting that these 2 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 adiponectin activation of nCDase; (3) Cav1 interacts with nCDase in a different fashion than AdipoR1. Whereas the Cav1/AdipoR1 interaction seems static, the Cav1/nCDase interface is dynamically regulated in an AdipoR1-dependent fashion; and (4) treatment

with adiponectin significantly brought Cav1/nCDase in closer proximity to each other. Combs et al⁴⁶ previously reported that mice overexpressing adiponectin displays elevated Cav1 levels in adipocyte and that Cav1-KO significantly reduces plasma adiponectin levels.⁴⁷ Collectively, currently available experimental evidence supports that Cav1 plays essential roles in adiponectin production as well as adiponectin transmembrane signaling.

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

Two limitations exist in the current study. First, we have provided clear evidence that adiponectin activation of AdipoR1 increases nCDase interaction with Cav1. However, how activation of AdipoR1 by adiponectin increases Cav1/nCDase interaction remains unclear. Because AdipoR1 directly interacts with Cav1, it is possible that adiponectin binding to AdipoR1 may promote Cav1 conformational change as that caused by other Cav1 interacting molecule,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/antioxidative/antinitrative actions of adiponectin are largely mediated by AdipoR1, not AdipoR2. In addition to these 2 evolutionarily acquired specific receptors, other cell surface binding proteins (such as T-cadherin) tether high molecular weight isoforms of adiponectin to the cell surface, possibly facilitating adiponectin/AdipoR interaction.⁵⁰ The role of these proteins in adiponectin's anti-inflammatory and vasculoprotective actions warrants future investigation.

In summary, we demonstrate for the first time that adiponectin inhibits TNF α -induced inflammatory response via Cav1-mediated ceramidase recruitment and activation in an AdipoR1-dependent fashion. These experimental results not only deepen our understanding of adiponectin 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 patient with diabetes mellitus.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Type 2 diabetes mellitus affects >20 million people in the United States and is a major risk factor for cardiovascular diseases.
- Adiponectin (APN), produced by adipocytes, protects against cardiovascular injury from inflammation, through interaction with one or both of its receptor proteins (AdipoR1 or AdipoR2).
- Adiponectin has been shown to decrease inflammation by activating ceramidase. It is, however, unknown whether adiponectin protects against vascular injury from inflammation by activating ceramidase or if AdipoR1/AdipoR2 themselves function as ceramidases.

What New Information Does This Article Contribute?

- Adiponectin decreases inflammation primarily through AdipoR1.
- The activation of ceramidase significantly contributes to adiponectin's anti-inflammatory function.
- The cell-signaling carrier protein caveolin-1 is essential for AdipoR1-mediated ceramidase activation and the resultant anti-inflammatory actions of adiponectin.

Patients with both type 1 and type 2 diabetes mellitus experienced more cardiovascular effects of inflammation than healthy

individuals. Inflammation plays a major role in the cardiovascular complications of diabetes mellitus. 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 adiponectin decreases inflammation through the enzyme ceramidase; however, it remains unknown whether adiponectin protects against vascular injury from inflammation by activating ceramidase or if AdipoR1/AdipoR2 themselves function as ceramidases. Here, we show that adiponectin decreases inflammation primarily through AdipoR1. Ceramidase activation significantly contributes to the anti-inflammatory effects of adiponectin. We demonstrate the necessity of caveolin 1 for AdipoR1-mediated ceramidase activation and the resultant anti-inflammatory actions of adiponectin. Our work contributes to a better understanding of adiponectin 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.

Supplemental Material

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 (rhTNF α) and nCDase antibody were from R&D System (Minneapolis, MN). Recombinant human globular and full length APN (gAPN and fAPN) were from Peprtech, Inc (Rocky Hill, NJ). S1P and SEW2871 (a selective S1P receptor-1 agonist²¹) 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 μ g/ml streptomycin at 37°C and 5% CO₂. Upon 80% confluence, cells were treated with vehicle, gAPN (2 μ g/ml¹⁴) or fAPN (10 μ g/ml). One hour after APN treatment, 10 ng/ml rhTNF α ²² was added. Cells were collected 12 hours after TNF α 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-1²³, AdipoR1²⁴, AdipoR2²⁴, AMPK α 1²⁴, and nCDase²⁵ 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 reagent 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: DGIWKASFTTFTVTKYWFYR; Alanine mutagenesis region: DGIWKASATTA AVTKYAAAYR. 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 2 μ g 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 μ l 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 μ l sample containing identical protein amount, 75 μ l 25 mM phosphate buffer (pH 7.4), and 0.5 μ l 4 mM Rbm14-12 substrate solution in ethanol (final substrate concentration 40 μ M; 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 μ l methanol and 100 μ l 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, λ_{ex} 355 nm, λ_{em} 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 μ mol/L) in medium for 5 minutes at 37°C. The intensity of the fluorescence signal was analyzed using IPlab 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 μ l 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 using O-

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 (pH7.6, 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 5 μ M 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 Na₄P₂O₇ 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 Na₄P₂O₇ 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. Cleared lysates were then incubated with 2 μ g 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 described³¹. 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.