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High Glucose/High Lipids Impair Vascular Adiponectin Function via Inhibition of Caveolin-1/AdipoR1 Signaling Formation

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Abstract

Reduced levels of adiponectin (APN) contribute to cardiovascular injury in the diabetic population. Recent studies demonstrate elevated circulating APN levels are associated with endothelial dysfunction during pre-diabetes, suggesting the development of APN resistance. However, mechanisms leading to, and the role of, vascular APN resistance in endothelial dysfunction remain unidentified. The current study determined whether diabetes cause endothelial APN resistance, and by what mechanisms. Under high glucose/high lipids (HG/HL), APN-stimulated nitric oxide production by HUVEC was decreased, phosphorylation of eNOS, AMPK, and Akt was attenuated ($P < 0.01$), and APN's anti-TNF α effect was blunted ($P < 0.01$). APN receptor expression remained normal, whereas Cav1 expression was reduced in HG/HL cells ($P < 0.01$). The AdipoR1/Cav1 signaling complex was dissociated in HG/HL cells. Knock-down of Cav1 inhibited APN's anti-oxidative and anti-inflammatory actions. Conversely, preventing HG/HL-induced Cav1 downregulation by Cav1 overexpression preserved APN signaling in HG/HL cells. Knock-in of a wild type Cav1 in Cav1 knock-down cells restored caveolae structure and rescued APN signaling. In contrast, knock-in of a mutated Cav1 scaffolding domain restored caveolae structure, but failed to rescue APN signaling in Cav1 knock-down cells. Finally, AdipoR1/Cav1 interaction was significantly reduced in diabetic vascular tissue, and the

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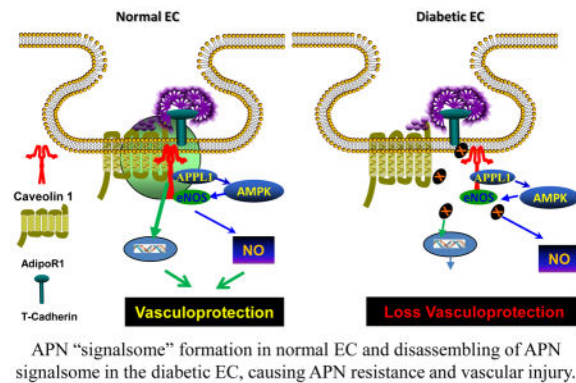
Disclosures

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vasorelaxative response to APN was impaired in diabetic animals. The current study demonstrates for the first time the interaction between AdipoR1 and Cav1 is critical for adiponectin-mediated vascular signaling. The AdipoR1/Cav1 interaction is adversely affected by HG/HL, due largely to reduced Cav1 expression, supporting a potential mechanism for the development of APN resistance, contributing to diabetic endothelial dysfunction.

Graphical abstract



Keywords

Caveolin; Adiponectin; Vascular Injury; Endothelial Function

Introduction

Cardiovascular disease (CVD) is the most significant cause of death in developed countries [18]. Despite extensive research worldwide in the past several decades, therapeutic interventions efficacious in reducing cardiovascular mortality remain limited. Type II diabetes is associated with atherosclerotic CVD [25]. Early interventions remain pivotal in preventing CVD. Endothelial dysfunction gives genesis to diabetic micro- and macro-vascular complications, the major causes of morbidity and mortality in diabetic patients [6]. Elucidating the specific mechanisms responsible for endothelial dysfunction in diabetes may yield viable targets for early therapeutic interventions.

Type II diabetes is characterized by a high glucose and high lipid (HG/HL) state [5]. HG/HL markedly increases oxidative/nitrative stress, which contributes centrally to diabetic endothelial dysfunction [12]. Additionally, chronic HG/HL induces a state of low grade inflammation in the vasculature, initiating inflammatory processes causing micro- and macro-vascular damage [1]. Concomitantly, systemic and vascular stress reduces endothelial nitric oxide (NO) bioactivity, and increases expression of cell surface adhesion molecules, ultimately augmenting atherosclerosis development. Constitutively expressed in endothelial cells, the intercellular adhesion molecule-1 (ICAM-1) is upregulated in human atherosclerotic lesions, which recruits leukocytes into the intima. As inflammatory cells migrate into the endothelium, proinflammatory cytokines (such as TNF- α) exacerbate atherosclerotic vascular disease [15,19,30].

Adiponectin (APN), a 30 kD protein of primarily adipose origin with multiple metabolic functions, protects the cardiovascular system against inflammation [2,11]. APN deficiency is associated with endothelial dysfunction and increased inflammatory responses in both diabetic rodent models and humans [16,22,24]. However, we recently demonstrated endothelial dysfunction occurs during early development of diabetes (pre-diabetes)[17]. Strikingly, plasma APN levels are significantly increased during this pre-diabetic stage. Moreover, there is no significant change in the expression levels of signaling molecules involved in APN vascular function, indicating the involvement of an unknown molecular mechanism. Interestingly, numerous recent epidemiological studies report elevated circulating APN levels in patients with heart failure, suggesting the onset of APN resistance with heart failure [3,8,14]. However, whether the metabolic derangements in pre-diabetes cause APN resistance (and are causatively related to subsequent endothelial dysfunction) has not been elucidated. Finally, we have recently demonstrated that AdipoR1 and Cav1 co-localize and co-precipitate in HUVECs, and the Cav1/AdipoR1 complex formation is critical for APN transmembrane signaling. However, whether this interaction is altered in diabetic condition, contributing to APN resistance and vascular injury, has not been previously investigated.

The objectives of the current study were 1) to determine whether HG/HL (mimicking the pre-diabetic stage) may cause APN resistance, and if so, by what molecular mechanisms, and 2) to assess potential therapeutic interventions capable of improving APN sensitivity and preserving endothelial function.

Materials and Methods

Materials

Human umbilical vein cells (HUVEC, 4–6 passages) and all cell culture reagents and medium were from Cell Applications (San Diego, CA). Cells were cultured in endothelial growth medium with 10% fetal bovine serum (Hyclone, Logan, CT), 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO₂. Antibodies against APN receptor 1 (AdipoR1), APN receptor 2 (AdipoR2), endothelial NO synthase (eNOS), AMP activated protein kinase (AMPK), Akt, ICAM-1, GAPDH, eNOS phosphorylated at Ser1177, phosphorylated AMPK, and phosphorylated Akt were from Cell Signaling Technology (Beverly, MA). Antibody to Cav1 was from Abcam (Cambridge, MA). Antibody against gp91^{phox} was from BioLegend Company (San Diego, CA). Antibody against Flag was from Sigma (St. Louis, MO). Antibody against AdipoR1 was from Bioss, Inc. (Woburn, MA) for Western analysis and IP.

HUVECs were randomized to receive one of the following treatments: Normal glucose/normal lipid culture for 72 hours (control, 5.5 mM D-glucose+19.5 mM L-glucose) followed by vehicle or APN (5µg/ml) treatment; or high glucose (HG, 25mM D-glucose)/high lipids (HL, 250 µM palmitates [23,28]) culture for 72 hours (HG/HL) followed by vehicle or APN treatment. The effect of HG/HL upon APN's endothelial stimulatory effect was determined by production of NO, and phosphorylation of eNOS, AMPK and Akt. The effect of HG/HL upon APN's anti-oxidative and anti-inflammatory actions was determined by superoxide production, peroxynitrite formation, and expression of gp91^{phox} and ICAM-1.

Small Interfering RNA Transfection

RNA oligonucleotides complementary to Cav1 target sequences silenced respective gene expression. HUVECs were transfected with a siIMPORTER siRNA transfection kit (Qiagen Science Inc. Benelux, Venlo, Netherlands) per manufacturer's protocol with siRNA duplexes against Cav1 (5'-UAUUAUGAGAUGGUAGGCAdTdT-3') and universal control oligonucleotides (AllStars, Westerville, OH). Briefly, HUVECs were plated on six-well plates before transfection. After reaching 80% confluence, siRNA was applied to each well (final concentration 50 nM).

Plasmid Construction and Transfection

The cDNA encoding full-length (FL) human Cav1 and scaffolding domain mutated Cav1 (muCav1, in which 6 aromatic residues within the scaffolding domain responsible for Cav1 interaction with its partner proteins were converted to alanine [21]) were subcloned into pcDNA3.1 at Hind III and XbaI sites. Full length adiponectin receptor 1 (AdipoR1) protein was generated using wild type AdipoR1 cDNA (gift from Liu/Dong Labs, Department of Pharmacology, University of Texas Health Science Center, San Antonio, TX) as a template. Truncated protein plasmids were additionally generated: AdipoR1 (1-230), AdipoR1 (1-215), AdipoR1 (316-375), and AdipoR1 (336-375) were generated via PCR and cloned into pcDNA3.1. Cav1 full length (FL), Cav1 (1-101), Cav1 (1-81), Cav1 (1-61) were generated via PCR using wild-type Cav3/Cav1 cDNAs as a template, and cloned into p3XFLAG.

HUVECs in a 60-mm culture plate were transfected with 2µg plasmid DNA encoding muCav1 and Cav1 FL via LipofectAMINE (Qiagen) per manufacturer's protocols. When cells were co-transfected with plasmid DNA, empty vector (no cDNA insert) served as control. Approximately 30 hours after transfection, culture medium was switched to 1% DMEM culture medium. Incubation commenced for 10 hours prior to any experiments.

HUVECs were grown to 90% confluence in 6-well dishes, and transfected by Lipofectamine 2000 Reagent. Specifically, DNA (2 µg of AdipoR1 expression vector, 2 µg of Cav1 expression vector, or empty vector: p3XFLAG, pcDNA3.1) was mixed with 10 µl transfection reagent (diluted in 100µl of serum-free medium) per manufacturer's protocol. 293T cells were incubated with lipid-DNA complexes in serum-free DMEM for 8 hours. Following transfection, cells were twice washed with PBS, and cultured in 10% serum medium. Lysates were prepared for co-IP analysis.

Electron Microscopy

HUVECs were fixed overnight at 4°C in a solution of 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH7.4. After subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 hour at room temperature, and rinsed in deionized H₂O in preparation for block staining by 2% uranyl acetate. After dehydration by graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate, and evaluated by a JEOL 1010 electron

microscope (fitted with a Hamamatsu digital camera running AMT Advantage image capture software).

Co-Immunoprecipitation and Immunoblotting

After treatment, HUVECs or 293T cells were lysed with cold lysis buffer, homogenized, and analyzed. Briefly, cell lysates were pre-cleared with corresponding nonimmune IgG incubated together with protein A plus-Sepharose for 30 minutes at 4°C. Cleared lysates were then incubated with 2 µg of anti-AdipoR1, anti-Cav1, anti-Flag, or anti-Myc antibodies. Cell lysates were then incubated with protein A plus-Sepharose overnight at 4°C. Non-immune rabbit IgG served as negative control. Protein A beads were then extensively washed with lysis buffer. Proteins were eluted from the beads, resolved by elution buffer, and subjected to Western blot analysis.

Samples with 2XSDS sample buffer were heated and separated by electrophoresis. After transfer to PVDF membranes, proteins were immunoblotted with appropriate antibodies. Signals were quantified by densitometry. Results were expressed as ratios of phosphorylated to unphosphorylated protein, or normalized to GAPDH loading control.

Subcellular Fractionation by Sucrose Density Gradient Centrifugation: Whole hearts or caveolae-rich membrane fractions were isolated as previously described². Briefly, 150-mm dishes of endothelial cells were grown to confluence, scraped into 2ml of MBS (MES-buffered saline [25mM MES (pH6.5), 150mM NaCl, 2mM EDTA, 250 mM NaCO₃]) without detergent. Triton X-100 was replaced with sodium carbonate buffer. Samples were homogenized upon ice (three 3 second bursts at medium speed), sonicated (six 10-second pulses of 400 watts), and incubated on ice for 10 minutes with periodic tube inversion. The resulting cell lysates were mixed with equal amounts of 90% sucrose in MBS (end sucrose concentration 40%). Resulting lysates were transferred to a centrifuge tube, and overlaid with 4 ml each of 35% and 5% sucrose in MBS. The gradient was centrifuged at 39,000 rpm for 20 hours by a Beckman SW41Ti rotor at 4°C. Twelve 1-ml fractions were collected from the gradient bottom, and analyzed by Western blot analysis or immunoprecipitation. Fractions 4–6 were buoyant membrane fractions (BFs) enriched in caveolins and proteins associated with caveolins. Fractions 9–12 were nonbuoyant fractions (non-BFs).

Confocal Microscopic Analysis

Endothelial cells were fixed with 4% paraformaldehyde/PBS for 15 minutes and rinsed in PBS. They were then treated with primary antibodies (rabbit anti-mouse Cav1 or goat anti-mouse APNR1 antibody at 1:200 dilution), then tetramethyl rhodamine (TRITC)-conjugated anti-mouse IgG and Cy5-conjugated anti-goat IgG (both at 1:200 dilution). After washing with PBS, coverslips were mounted using an anti-fade solution (KPL, Gaithersburg, MD). Negative control slides without primary antibodies were also stained and processed. Fluorescent images were acquired by Fluoview software (Olympus) on a FV1000 confocal microscope (Olympus, Tokyo, Japan).

Determination of NO_x in Endothelial Cell Culture Medium

Endothelium-derived NO and its metabolic products (NO₂ and NO₃), collectively known as NO_x, were determined using a chemiluminescent NO detector (Sierra 280i, Boulder, CO, USA) as described in our previous study [26]. Briefly, samples of 50 µl of culture medium were injected into a gas stripping apparatus containing 2 ml of a 1% solution of vanadium (III) chloride in hydrochloric acid at 90°C which was connected to an NO analyzer. NO was measured according to the instructions of the manufacturer. The sensitivity of the NO analyzer is < 10 pmol/ml, with a linearity of 4 orders of magnitude. Calibrations were made according to the manufacturer's instructions with standard solutions of sodium nitrate and sodium nitrite (Sigma), respectively.

Animal model and Aortic Contractility and Relaxation Assay

All in vivo experiments were performed upon adult (8 weeks old) male C57BL/6 mice, in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals, and were approved by the Thomas Jefferson University Committee on Animal Care. Experimental mice were randomized to receive a high fat diet (HFD) (60 kcal%) (Research Diets Inc. D12492i) or a 10 kcal% control normal diet (ND, D12450Bi) containing the same protein content as the HFD. 10 weeks after ND or HFD, mice were anesthetized with 2% isoflurane, and descending aortic segments were isolated. Aortic segments were maintained into ice-cold Krebs-Henseleit (K-H) buffer consisting of (in mM): NaCl (118), KCl (4.75), CaCl₂·2H₂O (2.54), KH₂PO₄ (1.19), MgSO₄·7H₂O (1.19), NaHCO₃ (25), and glucose (10.0). Loose fat and connective tissue were removed, and 2–3 mm aorta rings were isometrically mounted upon a Multi-Wire Myograph System (DMT 620M, DMT-USA Inc, Ann Arbor, MI). Each aortic ring's resting tension was set at 4 mN, and maintained throughout the experiment. During the one hour equilibration, the rings were exposed to K-H buffer in the tissue bath (replaced every 15 minutes). Phenylephrine (10⁻⁹ to 10⁻⁶ M) was applied to evaluate contraction response. Upon achieving stable contraction, globular APN (0.3, 1, 10, and 30 µM) was administered to determine APN-induced vasorelaxative response. After ring response to cumulative APN administration stabilized, bath washout occurred, and re-equilibration commenced. The procedure was then repeated with 1–100 µM dose increments of acidified NaNO₂ (prepared by dissolving NaNO₂ in 0.1 N HCl titrated to pH 2.0), an agent directly inducing vasorelaxation. Data was obtained and analyzed by a Powerlab system (ADInstruments, Colorado Springs, CO).

Statistical analyses

Quantitative results are expressed as mean±SEM. Comparisons between two groups were analyzed by t test, comparisons among more than two groups were made by one-way analysis of variance, and comparisons between each group were made by post hoc analysis via Tukey test. P values less than 0.05 were considered statistically significant. All statistical analyses were performed via GraphPad Prism6.

Results

HG/HL treatment decreased APN signaling response in endothelial cells

Initiation and progression of endothelial dysfunction in type II diabetes is characterized by decreased bioavailability of endothelium-derived NO. Therefore, we determined whether HG/HL treatment altered APN signaling response in endothelial cells. In HUVECs cultured with control medium (Control), APN treatment increased NO production, enhanced eNOS phosphorylation at Ser 1177, increased AMPK phosphorylation, and enhanced Akt phosphorylation (Figure 1). Importantly, exposing HUVECs to HG/HL for 72 hours significantly blunted or virtually abolished APN signaling. Specifically, gAPN-mediated NO production and phosphorylation of eNOS, AMPK, Akt and VASP were all markedly blunted compared to APN response in HUVECs cultured with normal medium (decreased to 43.4%, 22.3%, 36.2%, and 29.1% of control cell response, respectively). These results demonstrate impaired endothelial NO production in response to APN in a condition mimicking diabetic pathology, initiating endothelial dysfunction.

HG/HL inhibits APN's anti-inflammatory effects

It is well known APN and TNF α exert reciprocal effects at multiple levels. Moreover, diabetes itself exacerbates inflammation, oxidative/nitrative stress, and increases TNF α production. However, whether the anti-TNF α effect of APN is impaired under diabetic conditions has not been previously investigated. HUVECs were cultured with normal culture medium or HG/HL medium in the presence or absence of TNF α . The effect of APN upon nitrotyrosine content and adhesion molecule expression was subsequently determined. As we and others have reported, TNF- α markedly increased nitrotyrosine production and ICAM-1 expression (Figure 2). After HG/HL administration, TNF- α further augmented nitrotyrosine production and ICAM-1 expression (1.22 and 1.27-fold, respectively). More importantly, whereas APN significantly decreased nitrotyrosine production and ICAM-1 expression in control HUVECs, HG/HL treatment reduced APN's inhibitive effect upon nitrotyrosine formation (50.2% inhibition), and virtually abolished its effect upon ICAM-1 expression (Figure 2A–2B). These results suggest HG/HL decreases APN's suppressive effect upon TNF- α induced inflammation and oxidative/nitrative stress.

Expression of APN signaling molecules are unaltered 72 hours after HG/HL culture, but interaction between AdipoR1 and Cav1 is significantly impaired

The results presented in Figures 1 and 2 demonstrate HG/HL impairs APN function, contributing to diabetic endothelial dysfunction and inflammatory response. To determine the molecular mechanisms leading to impaired APN function (APN resistance), we determined the expression levels of molecules known to be involved in APN signaling. Somewhat to our surprise, no significant changes were observed in the expression levels of AdipoR1, AdipoR2, APPL1, AMPK, or eNOS after 72 hours of HG/HL culture (Figure 2C), indicating mechanisms other than altered APN signaling molecule expression are responsible for impaired APN signaling in HG/HL-cultured HUVEC. We recently reported AdipoR1 directly interacts with Cav3 in adult cardiomyocytes, facilitating APN cardiac signaling [27]. However, whether AdipoR1 may form a signaling complex with endothelial-expressed Cav1 to facilitate endothelial APN signaling (and more importantly, whether this

interaction is altered by HG/HL) has not been previously investigated. To evaluate these possibilities, several experiments were performed.

Confocal image overlay reveals AdipoR1 and Cav1 reside in the same cell membrane region (Figure 3A). To further establish the relationship between AdipoR1 and Cav1, HUVEC lysates were immunoprecipitated with an antibody against Cav1 or nonimmune IgG. AdipoR1/Cav1 proteins were detected in precipitates via Western blot. As shown in Figure 3B, AdipoR1 was detected in lysates immunoprecipitated with an antibody against Cav1, but was not detected in lysates immunoprecipitated with a non-immune IgG. When analyzed in reverse (immunoprecipitating the lysates with an antibody against AdipoR1 first), Cav1 protein was detected by Western blot (Figure 3B). To confirm the caveolar distribution of the AdipoR1/Cav1 complex, a caveolae-rich detergent-resistant membrane fraction was prepared from HUVECs. As illustrated in Figure 3C, AdipoR1 and Cav1 proteins were associated with the detergent-resistant membrane fractions (fractions 5–7).

It has been demonstrated previously that membrane proteins interact with the caveolin scaffolding domain via a specific caveolin-binding motif ($\phi X\phi XXXX\phi$ or $\phi XXXX\phi XX\phi$, where ϕ is an aromatic residue)^{20, 21}. Sequence analysis revealed 2 potential caveolin-binding motifs in AdipoR1 (²²⁰FVPWLYYSF²²⁸ and ³²⁵FFPGKFDIW³³³). To determine whether AdipoR1 binds Cav1 via these specific motifs, vectors expressing Myc-tag full-length AdipoR1 or truncated AdipoR1 were co-transfected with Flag-tag full length Cav1 into HUVECs. A co-immunoprecipitation assay demonstrated Cav1 protein was detected in cell lysates expressing full length AdipoR1, truncated AdipoR1¹⁻²³⁰, or truncated AdipoR1³¹⁶⁻³⁷⁵, but not in cell lysates expressing truncated AdipoR1¹⁻²¹⁵ or truncated AdipoR1³³⁷⁻³⁷⁵ (Figure 3D). These results demonstrate AdipoR1 interacts with Cav1 via the specific Cav1 binding motif. To determine whether the Cav1 scaffolding domain (residues 81-101) is required for AdipoR1 binding, we co-transfected Flag tag labeled truncated Cav1 protein and Myc tag labeled full length AdipoR1 into 293T cells (Figure 3E). AdipoR1 protein was detected in cell lysates with full length Cav1 or truncated Cav1⁶¹⁻¹⁰¹ and Cav1¹⁻¹⁰¹, but not in cell lysates with truncated Cav1¹⁻⁸¹, signifying AdipoR1 specifically binds to the Cav1 scaffolding domain.

Cav1 signaling is required for APN's anti-inflammatory and anti nitrate/oxidative effect in HUVECs

To test whether Cav1/AdipoR1 interaction is altered in the diabetic condition, the effect of HG/HL upon Cav1/AdipoR1 complex formation was determined. A co-immunoprecipitation assay demonstrated markedly reduced interaction between Cav1 and AdipoR1 after 72 hours of HG/HL treatment. Detection of Cav-1/AdipoR1 interaction in reverse sequence confirmed a similar result (Figure 4B). To determine the mechanism underlying impaired Cav1/AdipoR1 interaction after HG/HL culture, the expression levels of Cav1 and AdipoR1 was determined. Interestingly, although AdipoR1 expression level remains unaltered, Cav1 expression decreased 63.5% after 72 hours HG/HL treatment (Figures 4C and 4D respectively).

To investigate whether reduced Cav1 expression and impaired Cav1/AdipoR1 interaction contributes to the loss of APN-induced anti-inflammatory and anti-nitrate/oxidative stress

when exposed to HG/HL, loss- and gain-of function approaches were employed by utilizing Cav1 siRNA or Cav1 plasmid. Cav1 knockdown (Figure 5C, Cav1) abolished the attenuating effect of APN upon TNF α -induced nitrotyrosine formation (Figure 5A), superoxide production (Figure 5B), gp91phox expression (Figure 5C/D), and ICAM-1 expression (Figure 5C/E). Conversely, when HUVECs overexpressing Cav1 were exposed to HG/HL for 72 hours, APN markedly stimulated eNOS and AMPK phosphorylation (Figure 6A/B). Moreover, APN's anti-TNF α effects (as determined by ICAM-1 expression and nitrotyrosine formation) were fully recovered (Figure 6C/D). These data suggest Cav1 is critical for APN-induced anti-nitrative/oxidative stress and anti-inflammatory response in endothelial cells.

Cav1 scaffolding domain facilitates adiponectin signaling transduction

The above results concerning the interaction between AdipoR1 and Cav1 suggest Cav1 plays an important role in downstream APN signaling transduction. To further distinguish adiponectin signaling transduction dependency upon caveolar structure or caveolin scaffolding-mediated protein-protein interaction, additional experiments were performed. First, HUVEC endogenous Cav-1 expression was knocked down via Cav-1 siRNA. 72 hours after Cav1 siRNA, a wild-type or mutated Cav-1 was knocked-in. 72 hours after Cav1 knock-in, caveolae structure was determined via electron microscopy. APN signaling was determined by AMPK and eNOS phosphorylation. Cav1 knockdown significantly inhibited caveolae formation, which was restored by knock-in of either wild-type or mutated Cav1 (Figure 7A). However, attenuated AMPK and eNOS phosphorylation in response to APN was only rescued by knock-in of wild type Cav1, not mutated Cav1 (Figure 7B–D). These data importantly indicate the protein-protein interaction mediated by Cav1 is more essential than cell membrane caveolar structure in mediating adiponectin signaling transduction.

AdipoR1/Cav1 interaction is reduced and vasorelaxative response to APN is virtually abolished in the pre-diabetic vessel

The in vitro experimental results presented above demonstrate AdipoR1/Cav1 interaction is critical in APN signaling, a function impaired by HG/HL incubation. To clarify the in vivo pathologic relevance of this in vitro finding, a high-fat-diet induced diabetic animal model was utilized. In aortic samples from ND mice, AdipoR1/Cav1 complex was clearly detected (Figure 8A, ND). This interaction was significantly diminished in aortic samples from HFD mice (Figure 8A, HFD). Moreover, Cav1 expression is significantly reduced 10 weeks after HFD (Figure 8B). More importantly, in aortic rings freshly isolated from ND mice, APN induces concentration-dependent vasorelaxation (Figure 8C/D, ND). At the maximal APN concentration studied (30 μ M), APN induced 51.2 \pm 6.9% vasorelaxation. However, APN-induced vasorelaxation was virtually abolished in aortic segments isolated from 10 week-HFD mice (Figure 8C, HFD). The vasorelaxative response to acidified NaNO₂ is normal in all aortic segments studied, indicating preserved smooth muscle function in all tested vascular segments.

Discussion

This study investigated the mechanisms of endothelial dysfunction associated with systemic adiponectin resistance, a phenomenon garnering interest given the current obesity epidemic and prevalent associated vascular disease and endothelial dysfunction.

Our study demonstrates high glucose/high lipid impedes adiponectin receptor signaling transduction, compromising APN's downstream vascular protective effects. The interaction between AdipoR1 and Cav1 is requisite for adiponectin-mediated vasculoprotection. HG/HL conditions interfered with AdipoR1/Cav1 interaction to such extent that exogenous APN supplementation could not restore vascular function to control standards. This finding is consistent with a recent clinical observation [13] and experimental study [29] that elevated plasma adiponectin fails to provide protection in diabetic patients and animals. We demonstrate Cav1 overexpression may restore the anti-inflammatory, anti-oxidative, anti-nitrative effects of APN in an endothelial environment subjected to HG/HL conditions. The vascular protective effects of adiponectin are not solely reliant upon its circulating concentration, but its intact signaling machinery. It is worth noting that Cav1 siRNA alone moderately increased nitrotyrosine content and superoxide generation (Figure 5B/C). Although we do not have detailed molecular mechanism yet, our results suggest that physiological level of Cav1 is important in maintaining anti-oxidative signaling under basal condition.

Significant clinical data supports diabetes as a disease of chronic inflammation and nitrative/oxidative stress. It is accepted that prolonged exposure of the vascular endothelium to elevated levels of circulating inflammatory mediators perturbs endothelial cell homeostasis, provoking endothelial dysfunction [20]. Nitrotyrosine and ICAM-1 are indices of nitrative/oxidative stress and inflammation, processes implicated in accelerated atherogenesis and plaque formation [4]. Both are markedly upregulated in the diabetic vascular endothelium [7,10]. Our study demonstrated hyperglycemia/hyperlipidemia altered the interaction between caveolin-1 and adiponectin receptor 1, deleteriously affecting the anti-inflammatory and vaso-relaxative effects of adiponectin, evidenced by increased nitrotyrosine and ICAM-1. Such results prove for the first time that adiponectin resistance during diabetes has intricate relationship with caveolin family derangement in the setting of unchanged adiponectin receptor function.

We demonstrate the anti-nitrative/oxidative/inflammatory effects of adiponectin are reduced in a pre-diabetic environment, contributing to adiponectin resistance. To further elucidate the responsible molecular mechanisms, caveolin-1 expression was determined during HG/HL pretreatment. Cav1 expression reached nadir after 72 hours HG/HL exposure. Interestingly, AdipoR1 levels remained unchanged throughout HG/HL treatment, evidence that loss of adiponectin's anti-inflammatory properties cannot be attributed to receptor expression alteration. Caveolins are integral membrane proteins regulating intracellular signal transduction via caveolin scaffolding domains [9]. We previously demonstrated the imperative role Cav3 plays in adiponectin signal regulation in cardiomyocytes [27]. In the current study, by employing an isolated fresh vessel ring experiment, we demonstrated adiponectin enhanced the vasorelaxative effect of acetylcholine, which is lost during HG/HL

conditions. In summation, we provide evidence Cav1 facilitates regulation of APN signaling at the plasma membrane of endothelial cells, and beyond.

The present study clarifies two novel aspects of the relationship between caveolin-1 and adiponectin receptor 1 in endothelial cells. Our experiments utilized truncated protein fragments to reveal the Cav1 scaffolding domain can interact with AdipoR1's caveolin-binding motif; we further demonstrated the interaction was abrogated by mutated Cav1. The necessity of intact Cav1-AdipoR1 interaction for advantageous APN downstream signaling is exhibited many times over in this study; the Cav1-AdipoR1 relationship maintains APN's attenuation of both TNF α -induced ICAM-1 expression and HG/HL-exacerbated nitrotyrosine production. The second novel finding concerns the facilitation of adiponectin signaling transduction by the Cav1 scaffolding domain, not caveolae itself. The caveolae structure does not modulate adiponectin signaling transduction- proper Cav1 protein expression establishes the foundation for efficient and complete adiponectin signaling pathway transduction.

In summation, Cav-1/AdipoR1 interaction is impaired by in vitro HG/HL exposure, or in vivo HFD. Dissociation of Cav1/AdipoR1 impairs APN signaling, contributing to APN resistance and vascular injury (Figure 8E). Bolstering Cav1 signaling may be a novel approach reducing vascular injury in diabetic patients.

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NONSTANDARD ABBREVIATIONS

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AMPK	AMP activated protein kinase
APN	Adiponectin
BFs	Buoyant membrane fractions
Cav1	Caveolin-1
CVD	Cardiovascular disease
eNOS	Endothelial nitric oxide synthase
HG/HL	High glucose and high lipid

HUVEC	Human umbilical vein cells
ICAM-1	Intercellular adhesion molecule-1
NO	Nitric oxide

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highlights

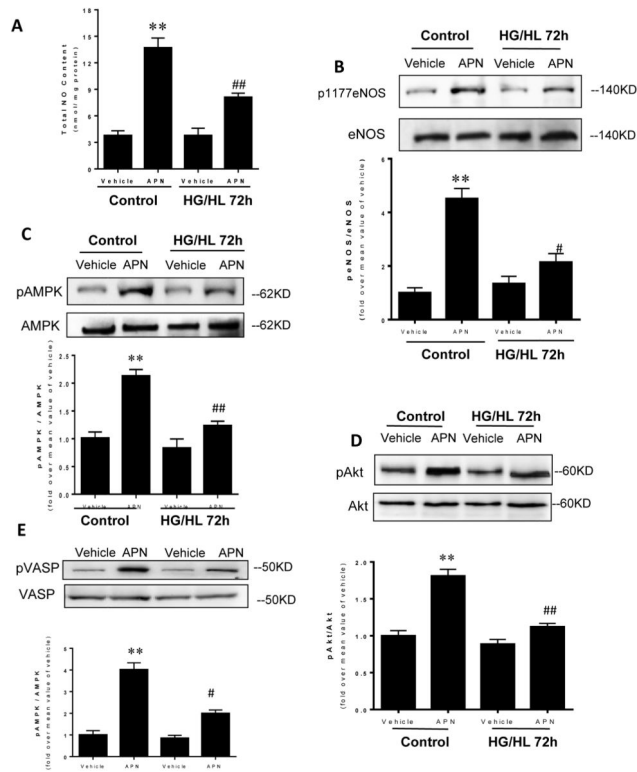
- The AdipoR1/Cav1 complex is critical for adiponectin signaling
- Diabetes downregulated Cav1 expression and separated AdipoR1/Cav1 complex
- Diabetes attenuates adiponectin's nitric oxide stimulatory and anti-oxidant actions
- Adiponectin resistance contributes to diabetic endothelial dysfunction

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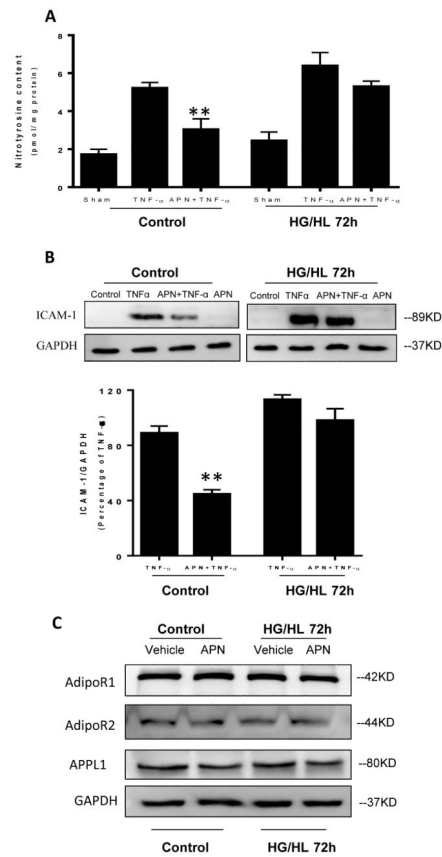
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**Figure 1.**

APN significantly increased NO production (chemiluminescent NO detector, A) and stimulated phosphorylation of eNOS (B), AMPK (C), Akt (D) and VASP (E) in control cells cultured with normal glucose/normal lipid (Control). After 72 hours of HUVEC exposure to HG/HL, APN biologic function was determined. NO production, eNOS, AMPK, Akt and VASP phosphorylation were significantly attenuated. N=6–8 dishes/group from at least 4 different cell batches. *P<0.05, **P<0.01 vs. respective vehicle control; ##P<0.01 vs. control group treated with APN.

**Figure 2.**

APN attenuated TNF- α induced nitrotyrosine formation (A) and ICAM-1 expression (B) in control cells cultured with normal glucose/normal lipid (Control). After 72 hours of HUVEC exposure to HG/HL, APN failed to reduce TNF- α mediated nitrotyrosine production (A) and ICAM-1 expression (B). No significant alteration was observed in expression levels of AdipoR1, AdipoR2 and APPL1 (C). N=6–8 dishes/group from at least 4 different cell batches. **P<0.01 vs. TNF α +Vehicle.

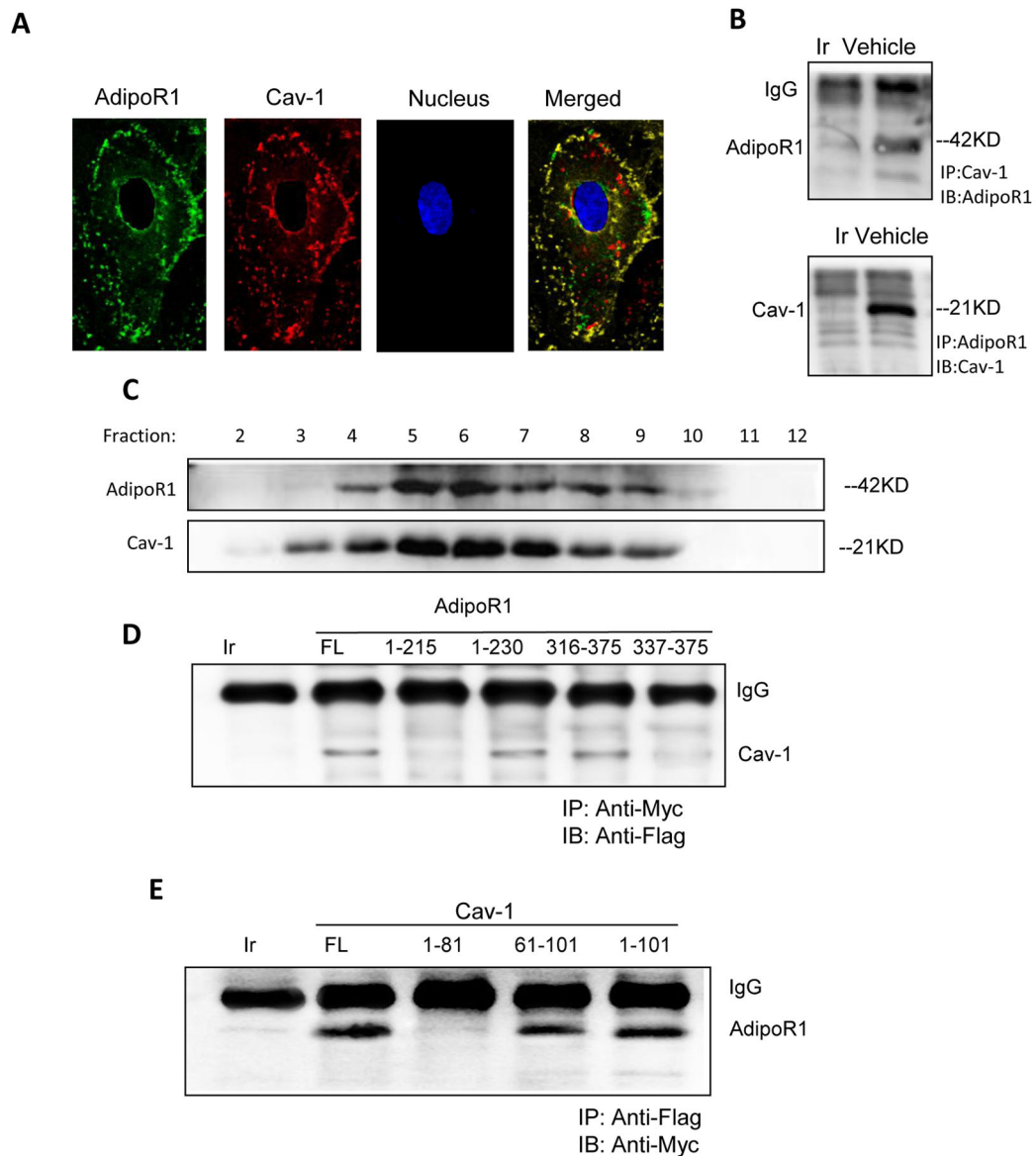
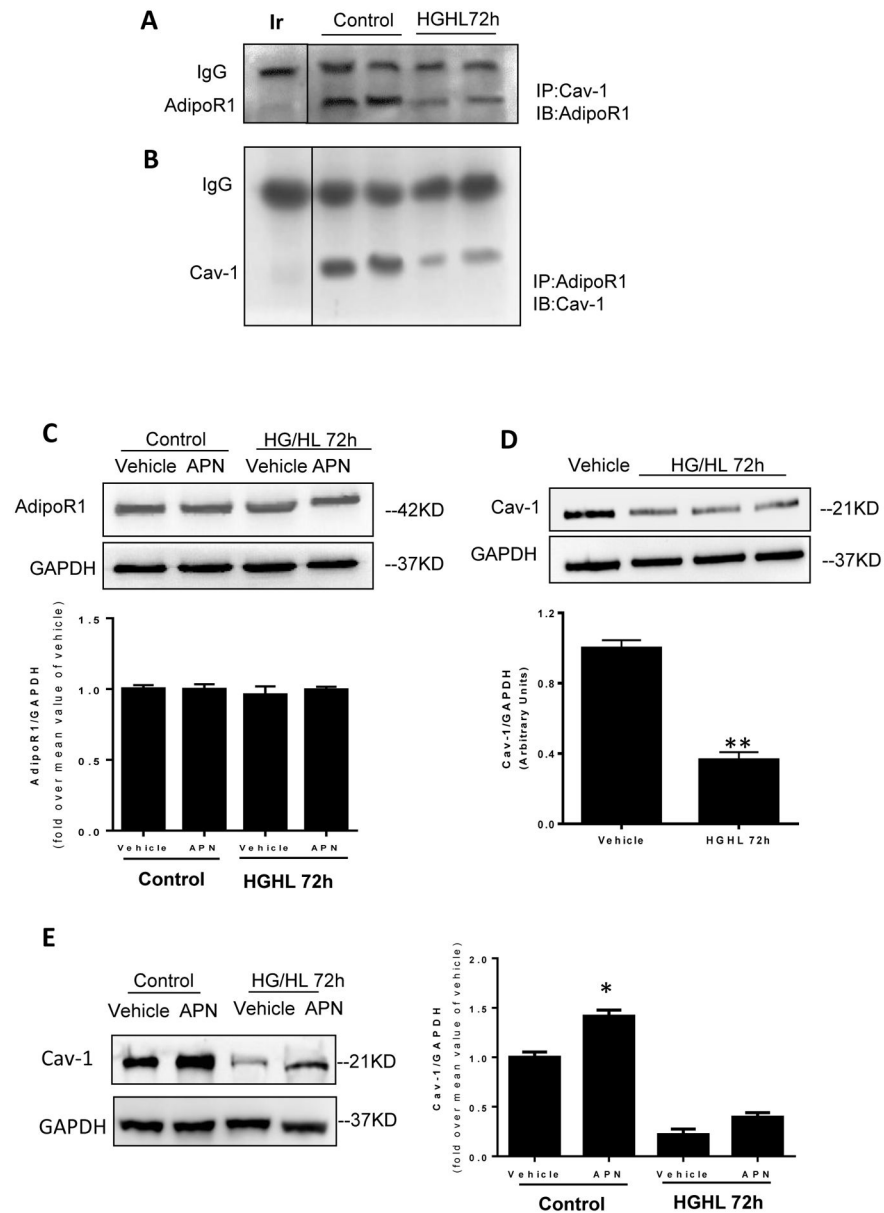


Figure 3.

Adiponectin receptor 1 (AdipoR1) co-localizes (A) and interacts with Cav1 (B). AdipoR1 was mainly distributed in the caveolae-rich fraction of detergent-resistant fractions (5–6), but seldom in detergent-soluble fractions (C). AdipoR1/Cav1 interaction was detected in HUVECs co-transfected with Cav1/AdipoR1 plasmid including full length AdipoR1 (FL), truncated peptide¹⁻²³⁰, truncated peptide³¹⁶⁻³⁷⁵, but not in cell lysates expressing truncated AdipoR1 peptide¹⁻²¹⁵ and truncated AdipoR1³³⁷⁻³⁷⁵ (D). Conversely, Cav1/AdipoR1 interaction was identified in HUVECs co-transfected with full length AdipoR1 and full length Cav1(FL), truncated Cav1 peptides⁶¹⁻¹⁰¹, truncated Cav1¹⁻¹⁰¹, but not in truncated Cav1¹⁻⁸¹ (E). N=4–5 dishes/group from at least 4 different cell batches. Abbreviations: Ir, irrelevant IgG; FL, full length; IP, immunoprecipitation; IB, immunoblot.

**Figure 4.**

AdipoR1 interacts with Cav1 to form a protein complex, an interaction markedly reduced when HUVECs were treated with HG/HL for 72 hours (A). Cav1 interacted with AdipoR1, an interaction decreased after 72 hours HG/HL treatment (B). AdipoR1 expression was unchanged after 72 hours HG/HL treatment of HUVECs (C). Cav1 expression was markedly decreased after 72 hours HG/HL treatment of HUVECs (D). N=6-8 dishes/group from at least 4 different cell batches. Abbreviations: Con, control. **P<0.01 vs. vehicle control.

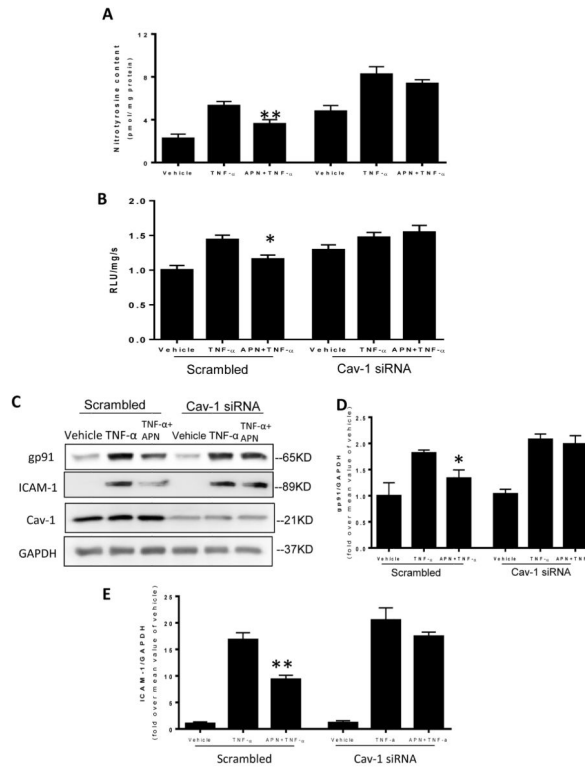
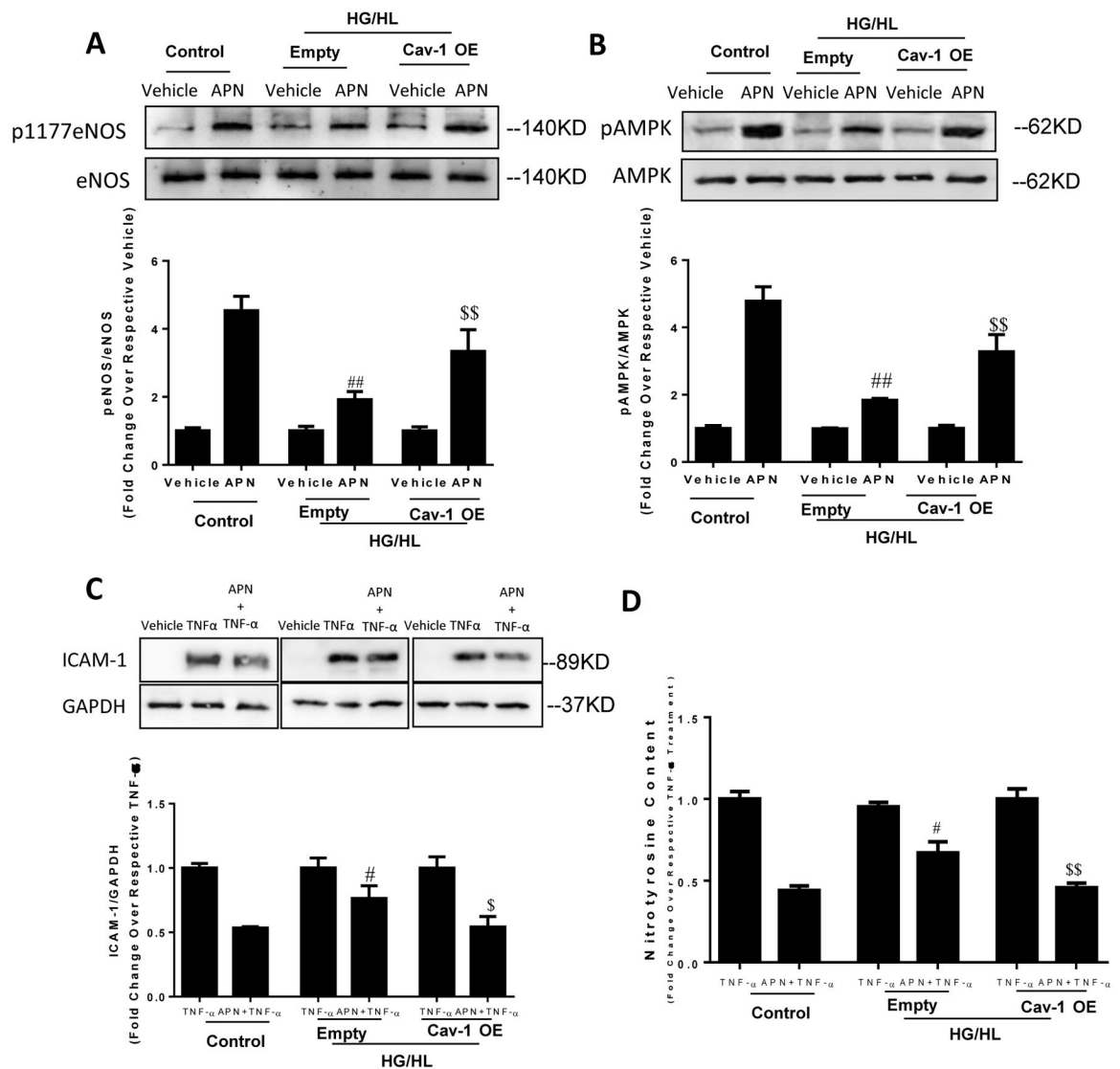
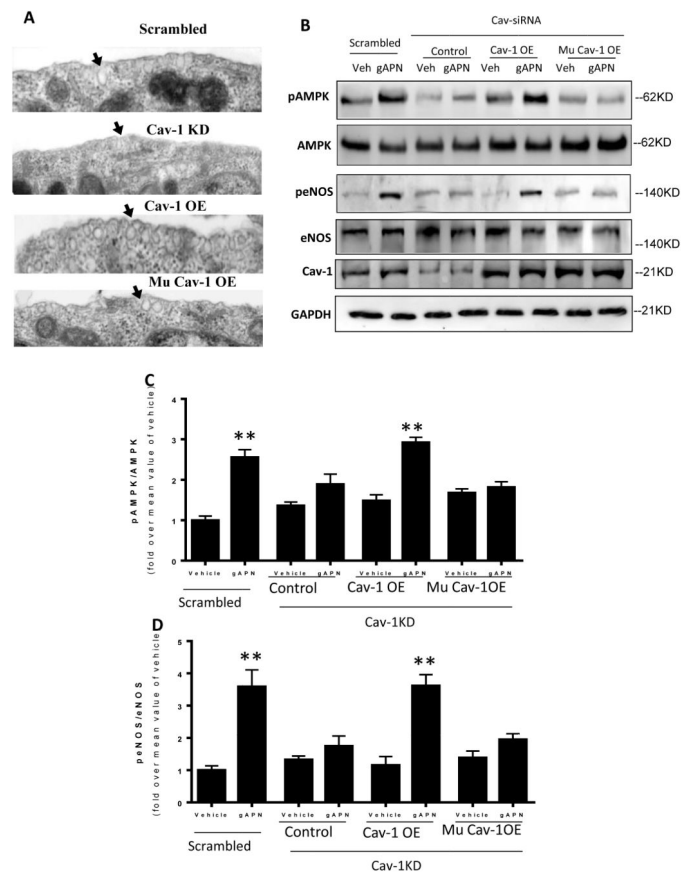


Figure 5. Cav1 knockdown abolished APN's anti-nitrate stress (determined by nitrotyrosine content) (A) and anti-oxidative stress (determined by superoxide generation) (B), and markedly blunted APN's anti-NADPH oxidase (gp91^{phox}) expression (C= representative blots; D=bar graph) and ICAM-1 expression (C= representative blots; E=bar graph) in HUVECs pretreated with gAPN 2 hours, and subjected to TNF- α (10 hours). N=6–8 dishes/group from at least 4 different cell batches. *P<0.05, **P<0.01 vs. vehicle control.

**Figure 6.**

Cav1 overexpression (Cav-1OE) preserved APN-induced eNOS and AMPK phosphorylation (A, B) in HUVECs cultured in HG/HL for 72 hours. Cav-1OE restored APN's anti-nitrative and anti-inflammatory effect (C/D) following TNF α treatment in HUVECs cultured with HG/HL medium. N=6–8 dishes/group from at least 4 different cell batches. #P<0.05, ##P<0.01 vs. APN+TNF α in control group (normal culture medium); \$P<0.05, \$\$P<0.01 vs. HUVECs transfected with empty plasmid (Empty).

**Figure 7.**

Knock-in of a wild type Cav1 (Cav1-OE) in Cav1 knock-down cells restored caveolae structure (A) and rescued APN signaling as determined by AMPK and eNOS phosphorylation (B, C, D). In contrast, knock-in of a mutated Cav1 scaffolding domain (Mu Cav1-OE) restored caveolae structure (A), but failed to rescue APN signaling in Cav1 knock-down cells (B, C, D). Arrows in panel A indicate caveolae structures in the cell surface. N=6–8 dishes/group from at least 4 different cell batches. **P<0.01 vs. respective control.

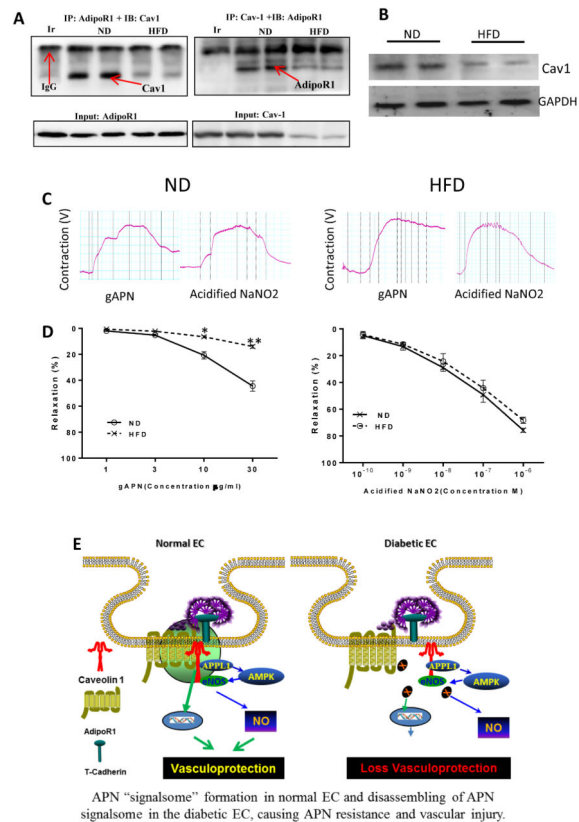


Figure 8.

High-fat-diet (10 weeks) induced diabetes impaired AdipoR1/Cav1 integration decreased Cav1 expression, and and blunted APN-induced vasorelaxation. (A) Aortic samples from ND (left two lanes) and HFD (right two lanes) were immunoprecipitated by antibody against AdipoR1, followed by immunoblotting with antibodies against Cav1 or immunoprecipitated by antibody against Cav1, followed by immunoblotting with antibodies against AdipoR1; (B) Cav1 expression was determined by Western blot analysis; (C) Original recording of APN-induced vasorelaxation in aortic segments isolated from ND and HFD mice. (D) Dose-response curves of APN-(left) and acidified NaNO₂-induced vasorelaxation in aortic segments isolated from ND and HFD mice (n=10–12 aortic segments/group from at least 4 animals/group). *P<0.05, **P<0.01 vs. ND group. Abbreviations: Ir, irrelevant IgG. (E) Graphic illustration of AdipoR1/Cav1 signalsome in APN transmembrane signaling under normal condition (left) and its impairment under diabetic condition (right).