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 (rhTNFα) and nCDase antibody were from R&D System (Minneapolis, MN). Recombinant human globular and full length APN (gAPN and fAPN) were from Peprotech, Inc (Rocky Hill, NJ). S1P and SEW2871 (a selective S1P receptor-1 agonist[21]) 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% fatal bovine serum, 2mM glutamine, 100U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. Upon 80% confluence, cells were treated with vehicle, gAPN (2 μg/ml[14]) or fAPN (10 μg/ml). One hour after APN treatment, 10 ng/ml rhTNFα[22] was added. Cells were collected 12 hours after TNFα treatment and oxidative/nitrative stress and ICAM-1 expression were determined as described in detail below.

Small Interfering RNA Transfection, Plasmid Construction, and Transfection

siRNA duplexes against Cav-1[23], AdipoR1[24], AdipoR2[24], AMPKα1[24], and nCDase[25] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Universal control oligonucleotides (AllStars) from Santa Cruz served as negative control. HUVECs (80% confluent) were transfected via siIMPORTER siRNA transfection kit (Qiagen Science Inc. Benelux) per manufacturer's protocol (final siRNA concentration: 50 nM). The cells were then incubated at 37°C with mixture of transfection regent and siRNA. After 5 hours, the cells in each well were replaced with fresh growth medium. At 72 hours after transfection, the cells transfected with control and experimental siRNA were used for experiments, harvested separately for extraction of total protein, and used for Western blot analysis or Immunoprecitipation 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:DGIWKASATTAAVTKYAAYR. Endothelial cells were maintained in culture with 10% fetal bovine serum DMEM medium (Cell Applications, CA). For most experiments, cells in a

60-mm culture plate were transfected with 2μg of plasmid DNA encoding muCav-1, FLCav-1, using LipofectAMINE (Qiagen) according to the manufacturer's protocols. When cells were co-transfected with plasmid DNA, empty vector (no cDNA insert) was used as control. Approximately 4 hours after transfection, culture medium was switched to 10% DMEM culture medium and incubation proceeded for 48 hours prior to the experiments. Then the cells will be harvested for extraction of total protein and used for the following experiments.

Ceramidase Enzyme Activity Assay

The nCDase enzyme activity was determined as previously reported, with minor modification[26]. Briefly, at experiment conclusion, cells were collected and washed twice with PBS. Cell pellets were resuspended in 100 μl 0.25 M sucrose solution, sonicated, and centrifuged at 15,000 g for 3 minutes. The supernatant was collected and protein concentration was determined. A 25 μl sample containing identical protein amount, 75μl 25 mM phosphate buffer (pH 7.4), and 0.5 μl 4 mM Rbm14-12 substrate solution in ethanol (final substrate concentration 40 μM; final ethanol concentration 1%) were loaded into each well of a 96-well plate. The same incubation mixture without supernatant served as negative control. The plate was incubated at 37°C for 1 hour without agitation. The enzymatic reaction was stopped by adding 25 μl methanol and 100 μl NaIO4 (2.5 mg/ml) in 200 mM glycine/NaOH buffer (pH 10.6) to each well. The plate was placed in a dark room for 1 hour. Fluorescent intensity was quantified via SpectraMax Microplate Reader (Molecular Devices, λex 355 nm, λem 446 nm).

Determination of Superoxide and Peroxynitrite Content

Superoxide content was quantified by lucigenin enhanced luminescence, and the cellular origin of reactive oxygen species was determined by dihydroethidium staining (DHE, Molecular Probes, Carlsbad, CA). Briefly, histological detection of superoxide anion in situ was performed using fresh-cultured endothelial cells stained with DHE (5μmol/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 μl of chemically synthesized $ONOO^-$ (concentration: 100-120 mM) to 3 ml of 0.04% (0.4 mg/ml) BSA in PBS. The amount of nitrotyrosine present in the peroxynitrite-treated BSA solution was measured at 430 nm using a spectrophotometer (Beckman DU 640, Fullerton, CA) and expressed as nanograms per milliliter. The stock solution of the peroxynitrite-treated BSA was diluted with PBS (final nitrotyrosine concentration, 0.75–75 ng/ml). These standard samples, along with samples from endothelial cells (protein concentration, 4 mg/ml) were applied to disposable sterile ELISA plates (Corning Glassworks, Corning, NY) and allowed to bind for 1 h at 37°C in a microincubator shaker (Teitec Co., San Jose, CA). After blocking nonspecific binding sites with 1% BSA in PBS, the wells were incubated for 60 min at 37°C with a rabbit polyclonal anti-nitrotyrosine primary antibody (Millipore) and subsequently for 60 min at 37°C with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000, Amersham Pharmacia Biotech, Inc. Piscataway, NJ). After washing the plates, the peroxidase reaction product was generated 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 H_2SO_4. 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 ddH_2O (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 5μM of $CaCl_2$ and cells were incubated with antibody for 2 h at room temperature. After 10 min washing with TBS under agitation, cells were incubated with florescence 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 $Na_4P_2O_7$ and cocktail protease inhibitor; For Cav-1 immnoprecipitation, 10mM Tris, pH 8.0/60 mM n-octyl β-D-glucopyranoside/150 mM NaCl/1 mM sodium orthovanadate/20 mM NaF/1 mM $Na_4P_2O_7$ and cocktail protease inhibitor][29, 30]. After homogenization and centrifugation, the supernatant was collected. For immunoblotting, proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were then incubated with primary antibodies (anti-ICAM-1, anti-nCDase, anti-ACC, anti-pACC, anti-AdipoR1, anti-Cav1, and anti-GAPDH) and HRP-conjugated secondary antibody. The blot was developed with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL). Bands were visualized by a Kodak Image Station 4000R Pro (Rochester, NY). For co-immunoprecipitation, cell lysates were pre-cleared with corresponding nonimmune IgG, and incubated together with protein A plus-Sepharose for 30 minutes at 4°C. Cleaned lysates were then incubated with 2 μg of either anti-Cav1 or anti-nCDase antibodies. Cell lysates were then incubated with protein A plus-Sepharose overnight at 4°C. Nonimmune rabbit IgG served as negative control. Protein A beads were then extensively washed with lysis buffer. Proteins were eluted from beads, and resolved by elusion buffer. Samples with 2XSDS sample buffer were heated and separated by electrophoresis. After transfer to PVDF membranes, proteins were immunoblotted with anti-AdipoR1 (for Cav1/AdipoR1 interaction) or anti-Cav1 (for Cav1/ceramidase interaction) as described above.

Intravital Microscopy Analysis of Leukocyte Rolling and Adhesion

Leukocyte rolling and adhesion was assayed in mesenteric post-capillary venules by intravital microscopy as we previously described[31]. 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: $\gamma=4.9\times8(V_{mean}/D)$, where D is the venule diameter.