Apolipoprotein E Enhances Endothelial-NO Production by Modulating Caveolin 1 Interaction With Endothelial NO Synthase

Lili Yue, Jing-Tan Bian, Ivana Grizelj, Ana Cavka, Shane A. Phillips, Ayako Makino, Theodore Mazzone

Abstract—Apolipoprotein E (apoE) is widely expressed in mammalian tissues, and one of the important tissue-specific effects is the atheroprotection ascribed to macrophage-derived apoE in the arterial wall. However, underlying mechanisms are not well understood. In this study, using subcellular fractionation, confocal microscopy, and coimmunoprecipitation, we demonstrated that macrophage-derived apoE is internalized by endothelial cells and impacts the subcellular distribution/interaction of caveolin 1 (cav-1) and endothelial NO synthase (eNOS). The addition of apoE disrupts the heteromeric complex formed between cav-1 and eNOS, and increases NO production. Sterol and oxysterol enhance endothelial cav-1/eNOS interaction and suppress NO production, but these effects are reversed by apoE. Silencing endothelial cav-1 attenuates apoE-induced NO production, establishing the importance of the cav-1-eNOS interaction for the increment in endothelial NO produced by apoE. Consistent with these observations, macrophage-derived apoE significantly improves vasodilation to acetylcholine in resistance arteries isolated from adipose tissue of obese humans. We conclude that macrophage-derived apoE enhances endothelial NO production by disrupting the inhibitory interaction of eNOS with cav-1. These results establish a novel mechanism by which apoE modulates endothelial cell function.

Key Words: apolipoprotein E ■ endothelium ■ caveolin 1 ■ macrophages ■ NO ■ NO synthase

Apolipoprotein E (apoE), primarily produced in hepatocytes, circulates as a surface component of human very low-density lipoprotein and high-density lipoprotein and plays a major role in directing the systemic metabolism of these particles. However, apoE is also produced at a high level in extrahepatic tissues, for example, by macrophages and adipocytes. Macrophage-derived apoE, in particular, has been shown to be important in the vessel wall, where it contributes to atheroprotection. Vessel wall accumulation of monocyte-derived macrophages occurs early in atherogenesis, and the ability to modulate apoE concentration in response to local tissue-specific stimuli is likely important for vessel wall injury and physiological stress. Production and secretion of apoE by macrophages is substantially augmented by sterols and oxysterols. This increase in macrophage-derived apoE could be viewed as a protective response to the vessel wall injury caused by these agents.

Endothelial dysfunction, as manifested by impaired endothelium-dependent vasodilation, is well established as a marker of atherosclerosis, hypertension, and future cardiovascular events. Impaired production of NO by endothelial cells underlies impaired endothelium-dependent vasodilation, and factors that regulate such production have, therefore, been the target of intensive investigation. For example, apoE derived from transfected Chinese hamster ovary cells suppresses the activation of endothelial cells, most likely by stimulating activity of endothelial NO synthase (eNOS). An inhibitory interaction between the endothelial cell caveolin 1 (cav-1) and eNOS is thought to be an important factor regulating endothelial NO production, and stimuli that facilitate this interaction are associated with accelerated vascular disease. For example, hyperlipidemia enhances the cav-1-eNOS interaction and reduces endothelial NO production.

We recently discovered apoE as a regulator of cav-1 distribution in adipocytes. ApoE colocalizes with cav-1 in adipocytes, and an N-terminal portion of apoE enriched in aromatic amino acids displays affinity for the cav-1 scaffolding domain. The cav-1 scaffolding domain is also the proposed site for cav-1 interaction with eNOS. Endothelial cells express high levels of cav-1 but do not express apoE. In view of the importance of macrophage-derived apoE for preserving vessel wall homeostasis, the previously demonstrated...
favorable effect of exogenous apoE for suppressing endothelial cell activation, and the proximity of macrophages to endothelial cells in the injured vessel wall, we examined whether macrophage-derived apoE is taken up and retained by endothelial cells, whether internalized apoE impacts endothelial cell cave-1 subcellular distribution, and what impact macrophage-derived apoE would have on the cave-1-eNOS association. We further explored the functional relevance of macrophage-derived apoE on NO production in human endothelial cells and on NO-dependent vasodilation of resistance arteries isolated from humans.

Methods
Please see the online-only Data Supplement for the expanded Methods section.

Sodium Carbonate Extraction and Sucrose Gradient Isolation of Caveolae
The procedure to isolate human umbilical vein endothelial cell (HUVEC) caveolae was based on the detergent-free protocol, as described previously.

NO Production Measurement and Cav-1 Knockdown
The membrane-permeable fluorescent indicator diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein) diacetate (DAF-FM) was used as a probe to measure NO production. After removing the medium, the endothelial cells were loaded with 10 μmol/L of DAF-FM diacetate in M199 without phenol red for 30 minutes at 37°C and washed 3 times. After adding apoE-conditioned medium, intracellular NO change was measured using a Synergy HT microplate reader (BIO-TEK) with excitation and emission wavelength of 485 nmol/L and 528 nmol/L, for every 30 minutes at 37°C or as indicated.

Validated SureSilencing human cave-1 short hairpin RNA and control plasmids were purchased from SA Biosciences (Frederick, MD). Early passages of HUVECs were transiently transfected with short hairpin RNA using TransPass HUVEC Transfection Reagent (New England BioLabs, Ipswich, MA) following the manufacturer’s protocols. Seventy-two hours posttransfection, the combined RNA/protein isolation was performed using the NucleoSpin RNA/Protein kit (Macherey-Nagel) according to the manufacturer’s instructions. The knockdown of cave-1 was measured by RT-PCR and Western blot.

Study Subjects
Visceral fat was obtained from subjects (woman; BMI 48±12 kg/m²; n=12) undergoing bariatric surgery (Table S1, available in the online-only Data Supplement). Subjects with medical conditions were excluded. The study was approved by the University of Illinois Institutional Review Board, and all of the subjects gave written informed consent. Resistance arteries were cleaned of fat and connective tissue and prepared for measurement of vessel diameter, as described previously. Male apoE knockout and age-matched C57BL/6J mice were purchased from Jackson Laboratory. All of the animal protocols were approved by the institutional animal care and use committee of the University of Illinois at Chicago.

Statistical Analysis
Data are expressed as mean±SD. Dilation to acetylcholine is expressed as a percentage of maximum response to papaverine (10−4 m). Vasodilator responses were assessed with a repeated-measured ANOVA. Comparisons were performed using the 1-way ANOVA. Differences were judged to be significant at the level of P<0.05.

Results
Macrophage-Derived ApoE Is Taken Up and Retained by Endothelial Cells and Modulates the Subcellular Distribution of Cav-1 and eNOS
Using RT-PCR and Western blot, no endogenous apoE was detected in HUVECs or mouse endothelial cells (data not shown). Although apoE circulates at ≈5 mg/dL in the plasma, macrophage-derived apoE may be most important for defending normal vessel wall homeostasis. We used the J774 macrophage cell line (which does not produce endogenous apoE) and transfected it to constitutively secrete a physiological level of human apoE3 (J774E cells). As a control, we used J774 cells transfected with a G418 resistance construct only (J774C cells). After a 2-hour incubation in J774E-conditioned medium, intact apoE was easily detectable in the endothelial monolayer by Western blot (data not shown).

To evaluate the subcellular distribution of the exogenously added apoE in endothelial cells, and if this addition affected endothelial cave-1 and eNOS subcellular distribution, we next performed sucrose gradient fractionation (Figure 1). After incubation in J774C-CM (Figure 1A) without added cholesterol (CH; Figure 1A, top), cave-1 was distributed in lighter-density membrane fractions 4 and 5, as well as in denser fractions 8 through 11. eNOS was distributed throughout the gradient. The addition of CH to cells incubated in J774C-CM led to enrichment of cave-1 in the lighter density membrane fractions 3 through 5 and shifted eNOS into the lighter-density membrane fractions containing cave-1, thus leading to greater colocalization of these proteins in the light-membrane caveolar fraction. After incubation in J774E-CM (Figure 1B), apoE was detected in high-density cell fractions, and both eNOS and cave-1 appear shifted out of lighter-density membrane fractions and into higher density cell fractions. J774E-CM also suppressed the shift of cave-1 and eNOS into lighter density membrane fractions that was produced by CH. Figure 1C quantitates these observations by presenting the ratio of cave-1:eNOS in low-density membrane to high density membrane fractions. In both the absence and presence of CH, apoE reduced the colocalization of eNOS with cave-1 in light-density caveolar fractions.

We next used confocal microscopy to evaluate the subcellular localization of apoE, endogenous cave-1, and eNOS in endothelial cells (Figure S1, available in the online-only Data Supplement). In the top panels of Figure S1A, endothelial cells stained for cave-1 and eNOS after incubation in J774C-CM are shown. Cave-1 and eNOS are detected throughout cells, and the only colocalization is observed in the perinuclear region. Consistent with the results of Figure 1A, there is significant new colocalization at the endothelial cell plasma membrane after incubation with CH. In the bottom panels, after incubation in J774E-CM, there continues to be a small amount of colocalization of cave-1 and eNOS in the perinuclear region, but the colocalization at the plasma membrane after incubation in CH is suppressed, consistent with the results of the subcellular fractionation. The degree of colocalization of Cave-1 and eNOS normalized to that present in J774C-CM without CH is quantitated at the bottom panel.
of Figure S1A. In Figure S1B, staining for apoE and cav-1 shows that apoE distribution is largely intracellular and that apoE suppresses the ability of CH to shift cav-1 to the plasma membrane. Incubation with CH produces a substantial amount of intracellular colocalization of cav-1 and apoE.

ApoE Disrupts the Interaction of Cav-1 and eNOS

The above results suggest that apoE can disrupt the interaction between cav-1 and eNOS. The change in the colocalization of eNOS and cav-1 in response to incubation with macrophage-derived apoE was quantitatively evaluated using coimmunoprecipitation (Figure 2). For these experiments, endothelial cells were incubated in J774C or J774E-CM with or without added CH, as shown. After immunoprecipitation with the cav-1 antibody, the total amount of cav-1 was not changed by the different incubation conditions. CH increased coimmunoprecipitation of eNOS with cav-1 \( \times 1.7 \)-fold in J774C-CM (Figure 2B). However, the amount of eNOS coimmunoprecipitated with cav-1 was significantly reduced after incubation in J774E-CM in both the absence and presence of CH. Using apoE antibody (Figure 2C) led to coimmunoprecipitation of cav-1; however, no eNOS was detected in the complex. This result indicates that the cav-1 associated with eNOS is distinct from the cav-1 associated with apoE.

ApoE Regulates Endothelial Cell NO Production in a Cav1-Dependent Manner

We next determined whether apoE modulates NO production in HUVECs. As shown in Figure 3A, the addition of J774E...
leads to a time-dependent increase in basal NO production in HUVECs compared with the addition of J774C. The increased DAF-FM fluorescence is abolished by Nω-nitro-L-arginine methyl ester (L-NAME) for 30 minutes to block endothelial NO synthase (eNOS)–induced NO production. \( P < 0.01 \) vs J774C. B, HUVECs were transfected with control or cav-1 knockdown (1 Cav-1) short hairpin RNA (shRNA). After 72 hours, Cav-1 mRNA (top) and protein (bottom) levels were knocked down \( > 80 \% \). C, NO production induced by J774C-CM and J774E-CM after silencing Cav-1. \( P < 0.05 \) vs J774C. D, HUVECs were incubated alone or with cholesterol (CH; 40 \( \mu \)g/mL) or 15 \( \mu \)g/mL of 7-ketocholesterol (7KC) for 18 hours. Cells were then incubated with J774C or J774E-CM for 3 hours before measurement of NO production. The data represent the mean±SD of triplicate samples repeated in 3 independent experiments. \( P < 0.05 \), \( P < 0.01 \) vs J774C without cholesterol. E, Western blot analysis of eNOS comimmunoprecipitated with Cav-1 with the indicated additions. Total cellular eNOS and Cav-1 are also shown. F, ApoE restores acetylcholine–induced NO production in sterol-loaded cells. NO production was induced by stimulation with 100 \( \mu \)M of L-NAME for 60 minutes. Data represent the mean±SD from 3 different experiments. \( P < 0.05 \) vs unstimulated J774C. \( P < 0.05 \) vs stimulated J774C.

**Figure 3.** Apolipoprotein E (ApoE) stimulates NO production in human umbilical vein endothelial cells (HUVECs). A, Time course of NO production by endothelial cells during incubation with control (J774C) or apoE (J774E)-conditioned medium. Where indicated cells were also preincubated with 100 \( \mu \)M of L-NAME for 30 minutes to block endothelial NO synthase (eNOS)–induced NO production. \( P < 0.01 \) vs J774C. B, HUVECs were transfected with control or cav-1 knockdown (1 Cav-1) short hairpin RNA (shRNA). After 72 hours, Cav-1 mRNA (top) and protein (bottom) levels were knocked down \( > 80 \% \). C, NO production induced by J774C-CM and J774E-CM after silencing Cav-1. \( P < 0.05 \) vs J774C. D, HUVECs were incubated alone or with cholesterol (CH; 40 \( \mu \)g/mL) or 15 \( \mu \)g/mL of 7-ketocholesterol (7KC) for 18 hours. Cells were then incubated with J774C or J774E-CM for 3 hours before measurement of NO production. The data represent the mean±SD of triplicate samples repeated in 3 independent experiments. \( P < 0.05 \), \( P < 0.01 \) vs J774C without cholesterol. E, Western blot analysis of eNOS comimmunoprecipitated with Cav-1 with the indicated additions. Total cellular eNOS and Cav-1 are also shown. F, ApoE restores acetylcholine–induced NO production in sterol-loaded cells. NO production was induced by stimulation with 100 \( \mu \)M of L-NAME for 60 minutes. Data represent the mean±SD from 3 different experiments. \( P < 0.05 \) vs unstimulated J774C. \( P < 0.05 \) vs stimulated J774C.

ApoE Is Detectable in the Endothelium of Intact Vessels In Vivo

To confirm that apoE could be localized to endothelial cells in vivo, sections of aorta and heart were prepared from C57Bl/6 mice or apoE knockout mice (Figure S2) and stained for apoE and CD31 (an endothelial cell-specific marker). ApoE was detected only in wild-type mice. In both aortic and intra-myocardial vessels, apoE and CD31 colocalization were easily detectable (Figure S2).

ApoE Enhances Acetylcholine-Mediated Dilation of Intact Human Vessels

Increased endothelial NO production stimulated by apoE predicts enhanced endothelium-dependent vasodilation by apoE. To confirm this, we measured endothelium-dependent vasodilation in response to acetylcholine with or without perfusion of apoE in human resistance arteries isolated from the adipose tissue of obese humans. For these studies, internal arterial diameters were 124±45 and 67±18 \( \mu \)m before and after endothelin 1 exposure, respectively. As shown in Figure 4A, there was a significant increase in dilation to acetylcholine after exposure to J774E (n=12 subjects; Table S1). Nω-nitro-L-arginine methyl ester (an inhibitor of eNOS) abolished the vasodilation produced by J774E, indicating that the enhanced dilation depends on eNOS. In contrast, responses to the endothelium-independent vasodilator sodium nitroprusside were similar in both groups, confirming that the improved acetylcholine dilations in the presence of J774E are endothelium dependent and not related to changes in smooth muscle sensitivity to NO (Figure 4B).
Our results demonstrate a novel mechanism for the regulation of endothelial cell function by apoE. Macrophage-derived apoE is retained intact in endothelial cells where it modulates the subcellular distribution of cav-1, reduces the inhibitory influence of endogenous cav-1 toward eNOS, and increases eNOS-derived NO production. ApoE restores NO generation in sterol-loaded cells and regulates NO-dependent vasodilation in intact human resistance arteries. Although we cannot exclude the possibility that some of the impact of J774E-CM also derives from another factor secreted by macrophages working in concert with apoE, the importance of apoE is demonstrated by its direct association with cav-1 in coimmunoprecipitation experiments, and our recently reported data demonstrating affinity of the N-terminal portion of apoE for cav-1.16

ApoE knockout mice develop spontaneous hypercholesterolemia, and this, along with other factors, likely contributes to the impaired endothelium-dependent relaxation observed in this model.19,20 There are likely multiple mechanisms by which apoE influences endothelial function. For example, apoE promotes reverse CH transport, inhibits endothelial cell activation, and defends against oxidative stress.26 We now demonstrate that apoE suppresses the inhibitory interaction between cav-1 and eNOS and induces NO production by endothelial cells. We further show that apoE restores NO-dependent vasodilation of resistance arteries isolated from the adipose tissue of obese humans, which have been reported to have impaired vasodilation.27

In the endothelium, cav-1 regulates NO signaling by binding to and inhibiting eNOS activity.14 Increased cytosolic Ca2+/calmodulin or activation of the kinase Akt leads to eNOS activation and its dissociation from cav-1.28 Direct binding of eNOS to the scaffolding domain of cav-1 is a well-accepted mechanism for downregulating NO production, and stimuli that facilitate this interaction are associated with accelerated vascular dysfunction.13,14 Blood vessels from cav-1 knockout mice showed marked enhancement of the relaxation response to acetylcholine, which is reversed by treatment with N5-nitro-l-arginine methyl ester, a specific NO synthase inhibitor or rescued by reintroduction of cav-1.31,32

It is important to note that, whereas an antibody to cav-1 communoprecipitates both eNOS and apoE, an antibody to apoE communoprecipitates only cav-1 but not eNOS. This result suggests that the cav-1 associated with apoE is distinct from the cav-1 associated with eNOS and provides support for a model in which association of apoE with cav-1 competitively reduces the association of cav-1 with eNOS. We have shown previously that an N-terminal domain of apoE enriched in aromatic amino acids displays specific and saturable affinity for the cav-1 scaffolding domain.16 Therefore, we speculate that apoE association with the cav-1 scaffolding domain could displace eNOS, increase NO production, and thereby modulate vascular tone in a cav-1-dependent manner. ApoE suppression of the inhibitory interaction between cav-1 and eNOS may occur while both remain within plasma membrane domains or by shifting cav-1 to an intracellular location. For example, interrupting this interaction could involve partitioning of cav-1 between low-density and high-density domains of the plasma membrane.13,14 Alternatively, the 2 proteins could be separated between plasma membrane and higher-density intracellular membranes. The data in Figure 1 show that apoE does lead to a greater shift of cav-1 than eNOS out of low-density caveolar membrane fractions. The data in Figure S1 are consistent with the interpretation that at least a portion of the shifted cav-1 can be found in an intracellular location. Further evaluation of these 2 possibilities will require additional experimentation.

In summary, our data provide evidence of a novel role for apoE in modulating the cav-1-eNOS interaction in endothelial cells. Modulation of this inhibitory interaction by apoE influences NO production in endothelial cells and likely modulates NO-mediated vasodilation of intact human vessels. The effect of sterol or oxysterol to enhance cav-1/eNOS interaction and to thereby suppress NO production in endothelial cells. We further show that apoE restores NO-dependent vasodilation of resistance arteries isolated from the adipose tissue of obese humans, which have been reported to have impaired vasodilation.27
regulates endothelial function. Our findings establish that apoE modulates the cav-1-eNOS interaction and NO production by the endothelium. Taken together these data suggest that the regulation of the cav-1-eNOS interaction by apoE could provide a common mechanism for the pleiotropic effect of this protein across multiple mammalian tissue compartments and provide a novel candidate target pathway for modulating endothelial cell function in vivo.

Acknowledgment

We thank Stephanie Thompson for assistance with article preparation.

Sources of Funding

This work was supported by grant DK 71711 (to T.M.), grant DK 083506 (to A.M.), and grants HL 85614 and HL 095201 (to S.A.P.) from the National Institutes of Health.

Disclosures

None.

References


---

**Novelty and Significance**

**What Is New?**
- ApoE directly interacts with cav-1 in endothelial cells.
- ApoE disrupts the inhibitory actions of cav-1 toward eNOS.
- ApoE can increase NO production by endothelium and facilitates NO-dependent dilation of intact vessels.

**What Is Relevant?**
- NO is an endothelial-derived relaxation factor.
- NO relaxes blood vessels and regulates blood pressure.
- eNOS is the main source of vascular NO production.
- Inhibition of eNOS activity is linked to vascular dysfunction.

**Summary**

Our data provide evidence of an important role for apoE in modulating cav-1/eNOS interaction in the endothelium. ApoE increases NO production and restores NO-mediated vasodilation of intact human vessels. Our results provide a novel candidate target for modulating blood pressure.
Apolipoprotein E Enhances Endothelial-NO Production by Modulating Caveolin 1 Interaction With Endothelial NO Synthase
Lili Yue, Jing-Tan Bian, Ivana Grizelj, Ana Cavka, Shane A. Phillips, Ayako Makino and Theodore Mazzone

Hypertension. published online August 20, 2012;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2012/08/20/HYPERTENSIONAHA.112.196667

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/08/21/HYPERTENSIONAHA.112.196667.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ApoE Enhances Endothelial NO Production by Modulating Caveolin-1 Interaction with eNOS

Lili Yue, Jing-Tan Bian, Ivana Grizelj, Ana Cavka, Shane A. Phillips, Ayako Makino, and Theodore Mazzone,

1 Department of Medicine, University of Illinois at Chicago, Chicago, Illinois
2 Department of Physical Therapy, University of Illinois at Chicago, Chicago, Illinois

Running title: Yue: ApoE and endothelial caveolin-1

Correspondence to:
Theodore Mazzone, MD
Department of Medicine
NorthShore University HealthSystem
2650 Ridge Ave
Evanston, IL 60201
Ph. (847) 570-2510
Fax. (847) 570-2905
tmazzone@northshore.org

METHODS

Materials: Pooled human umbilical vein endothelial cells (HUVECs), endothelial growth medium (EGM)-2 were purchased from Lonza (Walkersville, MD). ExactaCruzTM A and C immunoprecipitation kits, cav-1 and β-actin antibodies from Santa Cruz (Santa Cruz, CA). Acetylcholine chloride (ACh), Nω-Nitro-L-arginine methyl ester (L-NAME), methyl-β-cyclodextrin, cholesterol (CH) and 7-ketocholesterol (7-KC) were from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 594 donkey anti-rabbit, Alexa Fluor 488 donkey anti-goat and HRP-conjugated donkey anti-mouse antibodies, DAF-FM (4-amino-5-methylamino-2'7'difluorofluorescein) diacetate were purchased from Invitrogen (Carlsbad, CA). Human apoE ELISA Kit was from MABTECH AB (Nacka Strand, Sweden). eNOS and CD31 antibodies were from BD Transduction (Franklin Lake, NJ).

Cell Culture: HUVECs were cultured in EGM-2 with 10% FBS and used between passages 3 to 5. HUVECs were plated at 6 × 10³ cells/well on 6-well tissue culture plates (Falcon, USA) and cultured in 2 ml EGM-2. When the cells were 80-90% confluent, the culture medium was replaced with serum-free EGM-2 for the time indicated in figure legends. J774 macrophages (which do not express endogenous apoE) were stably transfected to express a physiologic level of human apoE3 under the control of a CMV promoter along with a G418 resistance construct (J774-E cells). ApoE concentration in the medium was measured by ELISA kit (MABTE AB), according to the manufacturer’s instructions. These cells constitutively secrete 1–1.2 μg/ml of apoE over 24h, similar to the levels of apoE produced by primary cultures of human monocyte-derived macrophages. Control cells (J774-C) were transfected with a G418 resistance construct alone. No detectable apoE was secreted by these cells. The conditioned media were collected over 24h.
Co-Immunoprecipitation and Immunoblotting: HUVEC monolayers were washed with PBS and cell lysates were prepared in 50mM Tris (pH 7.4), 150 mmol/L NaCl, 0.25% sodium deoxycholate, 2mM EDTA, 1% Triton X-100 and protease inhibitor cocktail. Co-immunoprecipitation of apoE with cav-1 and eNOS, or cav-1 with apoE and eNOS were performed using the ExactaCruz™ A or C kits according the manufacturer’s instructions. Immunoprecipitated complexes were loaded onto 10.5-14% criterion gel for immunoblotting with apoE, cav-1 or eNOS antibodies. Bands were visualized using the corresponding horseradish peroxidase-conjugated ExactaCruz™ reagents and the enhanced chemiluminescence solutions per the manufacturer's specifications.

Sodium Carbonate Extraction and Sucrose Gradient Isolation of Caveolae: The procedure to isolate HUVEC caveolae was based on the detergent-free protocol as previously described. Briefly, cells were scraped into 2ml of 500 mmol/L sodium carbonate, homogenized with 15 strokes in a dounce homogenizer and then sonicated with one 20 sec burst at 35% energy. The homogenate was adjusted to 4 ml of 45% sucrose by adding 90% sucrose and placed at the bottom of an ultracentrifuge tube. The tube was then overlaid with 4ml of 35% sucrose and 4ml of 5% sucrose in buffer. The gradient was spun at 39,000rpm for 18h in an SW41 rotor. Twelve fractions (1ml each) were collected from the top (lower density) to the bottom of the tube. Each fraction was used for western blot.

Double Immunofluorescence Staining: HUVECs cultured on coverslips were fixed, permeabilized and blocked with Image-iT FX reagent and then further blocked with 2% BSA in PBS for 1h. The cells were then incubated with rabbit anti-cav-1, goat anti-human apoE or mouse anti-human eNOS antibodies in blocking buffer for 1h at room temperature. After washing three times, the cells were incubated with Alexa Fluor 594 conjugated donkey anti-rabbit (1:400), Alexa Fluor 488 conjugated donkey anti-goat (1:400) or Alexa Fluor 488 conjugated donkey anti-mouse (1:400) antibodies for 1h. The slides were mounted with ProLong Gold antifade reagent, and nuclei were counter stained with DAPI for examination by confocal microscopy (Zeiss 510 META, Jena, Germany).

Preparation of CH/Methyl-β-Cyclodextrin Complexes: Complexes were prepared using a minor modification of the method described by Christian. Briefly, a 242 ul of 10mg/ml cholesterol (CH) or 7-ketocholesterol (7-KC) in 100% ethanol was dried in a glass tube. Ten ml of cyclodextrin was added and the mixtures were vortexed, sonicated for 3 min and incubated overnight at 37°C. The CH to cyclodextrin molar ratio was 1:8.

Cryosection and Double Immunofluorescence Staining: Mice were fed a regular chow diet and used at 10 weeks of age. After euthanasia, the heart was removed, cut in half and washed in sterile PBS. The aorta was trimmed and injected with frozen tissue matrix (O.C.T.). The tissues were frozen in a bath with liquid nitrogen and 2 methyl butane. Frozen tissues were sectioned (10 μm thickness) at −20°C using a cryostat. Serial sections were collected on poly-L-lysine (Sigma, St. Louis, MO) coated glass slides, dried at room temperature and stored at −80°C. On the day of staining, sections were fixed in cold acetone and blocked with 1% BSA/PBS. The sections were then double immunofluorescence stained with apoE and CD31 (endothelial marker) antibodies. Thereafter, the sections were incubated using Alexa Fluor 488 donkey anti goat IgG and Alexa Fluor 594 donkey anti rabbit IgG as secondary antibodies in 1% BSA for 60 minutes and mounted in ProLong Gold antifade reagent with with DAPI.

Study Subjects: Obese woman (BMI 48 ± 12 kg/m², n=22) visceral fat were obtained from subjects undergoing bariatric surgery. Subjects with medical conditions were excluded. The demographics of study subjects are shown in table S1. The study was approved by the University of Illinois Institutional Review Board and all subjects gave written informed consent.
Reference List


Table S1. Characteristics of Study Population

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>Female (%)</td>
<td>100%</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>167 ± 5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>144 ± 28</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>136 ± 15</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>92 ± 16</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>139 ± 73</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>178 ± 27</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>113 ± 30</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>135 ± 14</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>74 ± 10</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; BMI, Body Mass Index; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein
Figure S1. Effect of apoE on eNOS and cav-1 localization in HUVECs. HUVECs grown on chamber slides were left untreated or treated with 40 ug/ml of cholesterol in serum-free medium over night, followed by an incubation in the indicated conditioned medium for 2h. Monolayers were then stained and imaged by confocal microscopy. A. Confocal images showing eNOS and cav-1 colocalization. The bars show eNOS and cav-1 colocalization as fold change compared to that observed in J774C-CM without cholesterol. Results are means ± S.D. (n=5). *P<0.05 **P<0.01. B. Cav-1 colocalization with apoE. Scale bar, 5 μM. Representative images from five independent determinations are shown.
Figure S2. ApoE is detectable in endothelial cells of intact vessels in vivo. Representative confocal images of aorta and an intramyocardial resistance arteriole from C57BL6/J or apoE KO mice stained with CD31 (red) or apoE (green). Nuclei were counterstained with DAPI (blue). Scale bars: 20 μm. Images are representative of at least 5 images collected per specimen. White arrow represents a magnified vessel wall portion showing overlapping CD31 and apoE staining.