ATP-Binding Cassette Transporter G1 and High-Density Lipoprotein Promote Endothelial NO Synthesis Through a Decrease in the Interaction of Caveolin-1 and Endothelial NO Synthase

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Abstract

Objective—To investigate whether cholesterol efflux to high-density lipoprotein (HDL) via ATP-binding cassette transporter G1 (ABCG1) modulates the interaction of caveolin (Cav) 1 and endothelial NO synthase (eNOS).

Methods and Results—ABCG1 promotes cholesterol and 7-oxysterol efflux from endothelial cells (ECs) to HDL. It was previously reported that ABCG1 protects against dietary cholesterol-induced endothelial dysfunction by promoting the efflux of 7-oxysterols to HDL. Increased cholesterol loading in ECs is known to cause an inhibitory interaction between Cav-1 and eNOS and impaired NO release. In human aortic ECs, free cholesterol loading promoted the interaction of Cav-1 with eNOS, reducing eNOS activity. These effects of cholesterol loading were reversed by HDL in an ABCG1-dependent manner. HDL also reversed the inhibition of eNOS by cholesterol loading in murine lung ECs, but this effect of HDL was abolished in Cav-1–deficient murine lung ECs. Increased interaction of Cav-1 with eNOS was also detected in aortic homogenates of high-cholesterol diet–fed *Abcg1*$^{-/-}$ mice, paralleling a decrease in eNOS activity and impaired endothelial function.

Conclusion—The promotion of cholesterol efflux via ABCG1 results in a reduced inhibitory interaction of eNOS with Cav-1.

Keywords

ABCG1; caveolin-1; cholesterol; high density lipoprotein; nitric oxide synthase

Endothelial dysfunction is a key feature of early atherosclerotic lesions in both human and animal models.[1–3] Plasma high-density lipoprotein (HDL) cholesterol levels are inversely

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related to the incidence of atherothrombotic disease.[4,5] A part of the atheroprotective effect of HDL may be related to its role in preserving endothelial function.[6,7] In humans, HDL levels are correlated with flow-mediated vasodilation responses of the brachial artery[6,7] and with decreased coronary vasoconstrictor responses.[8,9] HDL may have a specific role in reversing decreased endothelial NO synthase (eNOS) activity in human endothelial cells (ECs) treated with oxidized low-density lipoprotein (LDL)[10] or in reversing the decrease in eNOS-dependent vascular relaxation induced by high-cholesterol diets (HCDs).[11,12]

Two ATP-binding cassette transporters (ABCA1 and ABCG1) have a major role in inducing cellular cholesterol efflux[13–15] and are known to be expressed in ECs.[12,16] ABCA1 mediates cholesterol efflux to lipid-poor apolipoprotein (apo) A-1 but only modestly increases cholesterol efflux to HDL.[15,17,18] In contrast, ABCG1 promotes macrophage cholesterol efflux to HDL but not to lipid-poor apoA-1.[15,19–21] ABCG1 has a specific role in promoting the efflux of 7-oxysterols,[22] a dietary oxysterol that is detected at high levels in human atherosclerotic plaques and is abundant in oxidized LDL.[23] HDL treatment of human ECs prevents 7-oxysterol–induced reactive oxygen species production.[12] Furthermore, ABCG1 and HDL reversed a decreased level of the active dimeric form of eNOS by promoting the efflux of 7-oxysterols, resulting in protection against endothelial dysfunction in animals fed HCDs.[12]

Specific interactions between the cholesterol-binding protein caveolin (Cav) 1 and signal-transducing proteins, including eNOS and various kinases, are known to repress the catalytic activity of these enzymes.[24,25] Several studies showed that the increase in Cav-1 abundance induced by LDL cholesterol promotes its inhibitory interaction with eNOS, resulting in decreased NO production.[26] Cholesterol/oxysterol-mediated impairment of NO production may be involved in the pathogenesis of endothelial dysfunction induced by hypercholesterolemia and dietary sterols through a variety of mechanisms, including decreased eNOS expression, reduced eNOS dimer levels, or reduced eNOS substrate availability.[12,27,28] Indeed, by reducing circulating LDL cholesterol or directly inhibiting cholesterol synthesis in ECs, statins can reverse endothelial dysfunction. This may involve decreased Cav-1 expression and promotion of NO release through destabilization of the inhibitory Cav-1/eNOS interaction.[29] Endothelial Cav-1 also plays an important role in modulating eNOS activity in vivo and accelerates atherogenesis in *apoe^{−/−}cav-1^{−/−}* mice.[30] Although ABCG1 has a major role in inducing cholesterol efflux in ECs,[12] its role in reversing the inhibitory Cav-1/eNOS interaction has not been explored. The present study was undertaken to test the hypothesis that ABCG1, by promoting efflux of cholesterol from ECs, modulates the interaction of eNOS with Cav-1.

Methods

Materials

eNOS antibody was purchased from BD Transduction (Lexington, Ky); Cav-1 antibody from Cell Signaling (Danvers, Mass); and ABCG1 and scavenger receptor BI antibodies from Santa Cruz (Santa Cruz, Calif). *β*-Actin antibody, methyl-*β*-cyclodextrin, Ca^{2+} ionophore (A23187), cholesterol, 7-ketocholesterol, 7*β*-hydroxycholesterol, and 25-hydroxycholesterol were purchased from Sigma-Aldrich (St. Louis, Minn); and 27-

Hydroxycholesterol from Steraloids (Wilton, NH). HDL (density, 1.06–1.21 g/mL) was isolated by preparative ultracentrifugation, as previously described.[12] Methyl-β-cyclodextrin cholesterol (molar ratio, 2.5:1) was prepared as previously described.[31]

Cell Culture and Transfection

Human aortic ECs (HAECs)[12] and murine lung ECs (MLECs)[32] were cultured as previously described. All small and interfering RNAs (siRNAs) were purchased from Invitrogen (Carlsbad, Calif) and Santa Cruz (Santa Cruz, Calif). The HAECs or MLECs were transfected with siRNA using Lipofectamine RNAiMAX reagent from Invitrogen, as previously described.[12]

Immunoprecipitation and Immunoblotting

Mouse aortas were obtained from wild-type, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$, and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ mice as previously described.[12] Mouse aortas and ECs were homogenized in an octyl glucopyranoside–containing buffer (60-mmol/L octyl glucopyranoside, 50-mmol/L Tris-chloride [pH, 7.4], 150-mmol/L NaCl, 1-mmol/L EDTA, 1-mmol/L sodium orthovanadate [Na_3VO_4], and protease inhibitor cocktail from Roche [Indianapolis, Ind]). Homogenates were incubated with Cav-1 polyclonal antibody. After 16 hours at 4°C, magnetic beads coupled with protein G (Dynabeads Protein G from Invitrogen Dynal AS, Oslo, Norway) were added to the supernatant for a further 1-hour incubation at 4°C. Bound immune complexes were captured by the magnets (DynaMag-Spin; Invitrogen) and washed with octyl glucopyranoside buffer. The immunoprecipitates were eluted in Laemmli sample buffer from Biorad (Hercules, Calif).

Sodium Carbonate Extraction Followed by Sucrose Gradient Fractionation

A detergent-free method was used, as previously described.[33,34] Briefly, the cell homogenates prepared in hypotonic buffer (50-mmol/L sodium carbonate [Na_2CO_3]; pH, 11) were adjusted to 42.5% sucrose with 85% sucrose in 2-[morpholino] ethanesulfonic acid (Mes)-buffered saline (25-mmol/L Mes [pH, 6.5] or 150-mmol/L NaCl) and placed at the bottom of an ultracentrifuge tube. A 5% to 30% discontinuous sucrose gradient was formed and ultracentrifuged at 35 000 rpm for 16 hours in an SW40 rotor (Beckman, Palo Alto, Calif). Gradient fractions, 1 mL, were collected from the top of the tube to yield 12 fractions; and each fraction (fractions 1–12) was used for Western blot and cholesterol mass analyses.

Western Blotting and NOS Activity Assay

Aortic and cell lysates and the fractions obtained by sucrose gradient fractionation were processed for Western blotting, as previously described.[12]

The NO synthesizing activity was determined by quantifying the rate of the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline with commercially available kits (Calbiochem-Novabiochem, EMD Biosciences, La Jolla, Calif), as previously described.[12,35]

Statistical analysis was performed using the *t* test. Bonferroni posttests were also used. Results are represented as mean±SEM.

Results

Effects of Cholesterol and Oxysterols on Cav-1/eNOS Interaction in HAECs

A specific role of ABCG1 in the efflux of 7-oxysterols was previously reported[22]; also, 7-oxysterols, but not cholesterol, can induce eNOS dimer disruption.[12] To further evaluate the roles of HDL and ABCG1 on eNOS activity, we tested the effects of different oxysterols (ie, 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and cholesterol [each 40 μg/mL, except for 7-ketocholesterol and 7β-hydroxycholesterol, which were 10 μg/mL]) on eNOS activity in HAECs. Cholesterol and all tested oxysterols significantly decreased eNOS activity (Figure 1A, open bars). HDL treatment prevented the reduction of eNOS activity induced by cholesterol/oxysterols (Figure 1A, filled bars). The data suggest that the efflux of cholesterol and oxysterols have an important role in maintaining eNOS activity, although cholesterol loading did not affect eNOS dimer or phosphorylated eNOS levels.[12]

Previous studies have shown that high LDL cholesterol levels promote an inhibitory interaction of eNOS with Cav-1, resulting in a decrease in NO production.[26] Therefore, by reducing cholesterol and oxysterols in ECs, HDL might also reverse endothelial dysfunction by promoting cholesterol and oxysterol efflux and consequently increasing NO release through the destabilization of the inhibitory Cav-1/eNOS complex. To test this hypothesis, we incubated HAECs with cholesterol/oxysterols and HDL. The data demonstrated that cholesterol and oxysterols increased Cav-1/eNOS interaction (Figure 1B and C, open bars). HDL treatment reversed cholesterol/oxysterol-induced Cav-1/eNOS interaction (Figure 1B, filled bars). Total eNOS and Cav-1 levels were not affected by either cholesterol or oxysterols (Figure 1B).

Effects of HDL and ABCG1 on Cav-1/eNOS Interaction in HAECs

Next, we examined the effects of different concentrations of HDL on increased Cav-1/eNOS interaction and reduced eNOS activity by cholesterol loading in HAECs. HDL treatment reduced both the increase in Cav-1/eNOS interaction (Figure 2A) and the reduction in eNOS activity (Figure 2B) in a similar concentration-dependent manner (between 25 and 100 μg/mL). HDL treatment did not affect total eNOS or Cav-1 levels (Figure 2A). The incubation of HAECs with cholesterol and HDL simultaneously similarly reversed the Cav-1/eNOS interaction as sequential incubation with cholesterol and HDL (supplemental Figure I; available online at http://atvb.ahajournals.org).

To assess the role of ABCG1 in the ability of HDL to reduce Cav-1/eNOS interaction, we knocked down the expression of ABCG1 by siRNA. ABCG1 knockdown abolished the ability of HDL to reduce cholesterol-mediated Cav-1/eNOS interaction (Figure 2C) and the corresponding increase in eNOS activity (Figure 2D).

Effects of Cav-1 on eNOS Activity in MLECs

Next, we examined whether Cav-1 deficiency would abolish the sterol-mediated decrease in eNOS activity and the ability of HDL to activate eNOS. We used wild-type and *cav-1*$^{-/-}$ MLECs for these experiments because we could not achieve a greater than 50% Cav-1

knockdown in HAECs using several different Cav-1 siRNAs. Under basal conditions, we found that Cav-1 deficiency induced eNOS activity by approximately 100% compared with the controls (data not shown). Fernández-Hernando et al[30] found a similar increase in eNOS activity in *apoe*$^{-/-}$ *cav-1*$^{-/-}$ MLECs compared with *apoe*$^{-/-}$ MLECs. Because the eNOS activity in wild-type MLECs was relatively low under basal conditions, we added the Ca^{2+} ionophore (A23187) that mediates the dissociation of eNOS from Cav-1 to induce eNOS activity.[26] Therefore, the Ca^{2+} ionophore only increased eNOS activity in wild-type MLECs. In wild-type MLECs, cholesterol loading reduced eNOS activity, and this was completely reversed by HDL (Figure 3), similar to our observations in HAECs. In *cav-1*$^{-/-}$ MLECs, eNOS activity was not reduced by cholesterol loading and HDL had no ability to increase eNOS activity in cholesterol-loaded *cav-1*$^{-/-}$ MLECs (Figure 3). Thus, Cav-1 deficiency abolished the ability of HDL to increase eNOS activity.

eNOS, Cav-1, and ABCG1 Plasmalemmal Distribution in HAECs

Next, we examined the roles of HDL and ABCG1 on eNOS, Cav-1, and cholesterol plasmalemmal distribution in HAECs. eNOS was predominantly found in intermediate buoyant membranes (fractions 6–7), where it colocalized with Cav-1 in HAECs (Figure 4A). Free cholesterol (FC) loading caused a shift in eNOS and Cav-1 toward lighter-density fractions (fractions 4–6 and fractions 4–8, respectively), coinciding with FC distribution (Figure 4B). HDL treatment caused a redistribution of eNOS toward more dense fractions and away from Cav-1, especially in cholesterol-loaded cells, and a decrease in cholesterol content in cholesterol-loaded cells (Figure 4C and D). ABCG1 could be seen in heavy-density fractions, especially in cholesterol-loaded cells (fractions 9–12) and did not colocalize with eNOS or Cav-1 (Figure 4B). The distribution of ABCG1 and β-actin were not affected by either FC loading or HDL. To assess the role of ABCG1 in Cav-1/eNOS redistribution by HDL, we knocked down the expression of ABCG1 by siRNA (Figure 4E and F). In ABCG1 siRNA-transfected FC-loaded HAECs, the ability of HDL to cause redistribution of eNOS into more dense fractions and away from Cav-1 was markedly attenuated (Figure 4F). Methyl-β-cyclodextrin treatment, which removes plasma membrane cholesterol from raft/caveolae,[36] dramatically reduced cholesterol levels and caused a marked shift in eNOS and Cav-1 distribution into heavy-density fractions (fractions 8–12) (Figure 4G). These experiments suggest that HDL tends to dissociate Cav-1 and eNOS in the plasmalemma under FC-loading conditions only in the presence of ABCG1. Most likely, ABCG1-mediated cholesterol efflux to HDL explains these findings. However, ABCG1 does not colocalize with Cav-1 in gradient fractions.

eNOS, Cav-1, and ABCG1 Plasmalemmal Distribution in MLECs

We also examined the plasmalemmal distribution of ABCG1 in MLECs. In wild-type MLECs, Cav-1 was distributed into light buoyant membranes (fractions 1–4) and heavy membranes (fractions 8–12) (Figure 5A). eNOS was dominantly distributed into heavy membranes (fractions 8–12), and a small amount of eNOS could be seen distributed into light buoyant membranes (fraction 3) (Figure 5A). ABCG1 was found only in heavy-density fractions (fractions 9–12) (Figure 5A). In *cav-1*$^{-/-}$ MLECs, the distributions of ABCG1 and eNOS were unchanged compared with wild-type cells (Figure 5B).

Effect of ABCG1 on eNOS and Cav-1 Interaction in Mouse Aorta

It was recently documented that $Abcg1^{-/-}$ mice fed either an HCD or a Western-type diet exhibited a marked decrease in endothelium-dependent vasorelaxation and that this correlated with decreased levels of the active dimeric form of eNOS.[12] To further evaluate the role of ABCG1 in endothelial function, we investigated the impact of ABC transporter deficiency on eNOS and Cav-1 interaction, using samples from a previously published study.[12]

In response to the HCD, eNOS/Cav-1 interaction was dramatically increased (Figure 6, left). Total Cav-1 levels were increased by the HCD (Figure 6, left). Using the same samples, we observed in our previous study that total eNOS levels were slightly (approximately 28%) decreased in aortas from $Abcg1^{-/-}$ mice on the HCD.[12] Together with the data presented herein, this indicates that the ratio of Cav-1–bound eNOS, after correction for 28% decreased total eNOS, to total Cav-1 was increased in $Abcg1^{-/-}$ mice (Figure 6), paralleling the decreased eNOS activity in a previous study.[12] For the Western-type diet, $Abca1^{-/-}$, $Abcg1^{-/-}$, and $Abca1^{-/-}$ $Abcg1^{-/-}$ mice exhibited increased Cav-1–bound eNOS levels, whereas total Cav-1 levels were similar (Figure 6, right). Also, eNOS levels were similar as assessed in the same samples in a previous study.[12] The increase in Cav-1–bound eNOS levels was most prominent in $Abcg1^{-/-}$ and $Abca1^{-/-}$ $Abcg1^{-/-}$ mice (Figure 6, right), closely paralleling the decrease in eNOS activity previously found in arterial rings from these mice.[12]

Discussion

A principal antiatherogenic property of HDL is thought to be its ability to promote cholesterol efflux from macrophages; recent studies have highlighted the key roles of ABCA1 and ABCG1 in reversing macrophage foam cell formation[37] and atherosclerosis.[38,39] HDL has also exerted a variety of beneficial actions that are independent of macrophage cholesterol efflux. For example, HDL inhibits LDL oxidation, smooth muscle cell migration, and platelet aggregation; it also reverses endothelial dysfunction.[40–42] It was recently shown that ABCG1 and, to a lesser extent, ABCA1 help to preserve endothelial eNOS activity in mice fed HCDs.[12] The ability of ABCG1 to preserve endothelial function appeared to be at least partly related to its role in promoting the efflux of 7-oxysterols,[12] which are detected at high levels in human atherosclerotic plaques and are abundant in oxidized LDL.[23] This effect was associated with decreased reactive oxygen species formation and preservation of the active dimeric form of eNOS.[12] To further understand the beneficial effects of HDL and ABCG1 on endothelial function, the present study was undertaken to test the hypothesis that ABCG1, by promoting efflux of cholesterol and oxysterols from ECs, also modulates the inhibitory interaction of eNOS with Cav-1. We have shown that ABCG1 and HDL can reverse the Cav-1/eNOS interaction induced by cholesterol loading, resulting in increased eNOS activity.

Although 7-oxysterols have a prominent role in producing endothelial dysfunction, cholesterol accumulation also inhibits endothelium-dependent vasorelaxation.[11,12,43] Indeed, both FC and 7-oxysterols could increase the inhibitory interaction of eNOS with Cav-1 and could cause impaired NOS activity (Figure 1), whereas only treatment with 7-oxysterols led

to eNOS dimer disruption.[12] It was recently reported that ABCG1 is highly expressed in the endothelium of nonatherosclerotic aorta and showed accumulation of cholesterol and 7-oxysterols in ECs of high cholesterol diet–fed mice.[12] Figure 6 shows that the inhibitory interaction of Cav-1 with eNOS was increased in aortic homogenates of HCD or Western-type diet–fed *Abcg1* [−/−] mice, paralleling endothelial dysfunction.[12] Similar to some previous studies, we found an increase in Cav-1 levels in response to the HCD (Figure 6); however, we did not find such an effect in cholesterol-loaded HAECs. Thus, multiple different mechanisms can lead to the formation of inhibitory Cav-1/eNOS complex formation.

ABCG1 is highly expressed in HAECs,[16] and ABCG1 and HDL had a major role in promoting the efflux of cholesterol and 7-oxysterols from HAECs.[12] The expression of other proteins mediating cholesterol efflux, such as ABCA1 and the scavenger receptor BI, was low or downregulated by cholesterol treatment, respectively.[8] We found low levels of ABCA1-mediated efflux to lipid-free apoAI- or scavenger receptor BI–mediated cholesterol efflux to HDL in HAECs.[16] In this study, FC loading caused a shift in eNOS distribution into lighter-density fractions coinciding with FC distribution; this effect was reversed by HDL treatment in an ABCG1-dependent manner (Figure 4). ABCG1 distribution was distinct from Cav-1 (Figures 4 and 5), and cholesterol efflux to HDL via ABCG1 was not affected by Cav-1 expression (supplemental Figure II and supplemental Figure III). In addition, the distribution of ABCG1 was unaffected by FC loading. Taken together, our data suggest that the promotion of cholesterol efflux by ABCG1 leads to a redistribution of cholesterol from caveolae to noncaveolae domains, where ABCG1 localizes, consequently reducing caveolae cholesterol and reversing the inhibitory eNOS/Cav-1 interaction.

LDL cholesterol loading of ECs is known to increase the Cav-1/eNOS interaction, a process that was unaffected by treatment with antioxidants[26]; whereas eNOS dimer disruption, induced by 7-oxysterols, was reversed by antioxidants.[12] Reducing circulating LDL cholesterol and/or directly inhibiting cholesterol synthesis in ECs by statin could reverse endothelial dysfunction by decreasing inhibitory eNOS/Cav-1 interaction. Interestingly, our study indicates that a similar preservation of endothelial function can be exerted by HDL by promoting cholesterol or 7-oxysterol efflux via ABCG1. A number of different mechanisms have accounted for the ability of HDL to preserve eNOS activity,[12,27,28] and further studies will be required to determine their relative importance.

In humans, HDL levels are correlated with flow-mediated vasodilation responses of the brachial artery[6,7] and with decreased coronary vasoconstrictor responses.[8] The infusion of recombinant phospholipid/apoA-1 particles into Tangier disease heterozygotes with isolated low HDL levels reversed defective forearm blood flow measurements.[9] Therapies that increase HDL levels, such as niacin and cholesteryl ester transfer protein inhibitors, are probably activating the ABCG1-mediated cholesterol/oxysterol efflux pathway in ECs and macrophages. More important, niacin therapy has improved NO-mediated vascular relaxation in humans.[44] Our studies highlight the therapeutic potential of increasing HDL to correct endothelial dysfunction associated with dietary cholesterol and hypercholesterolemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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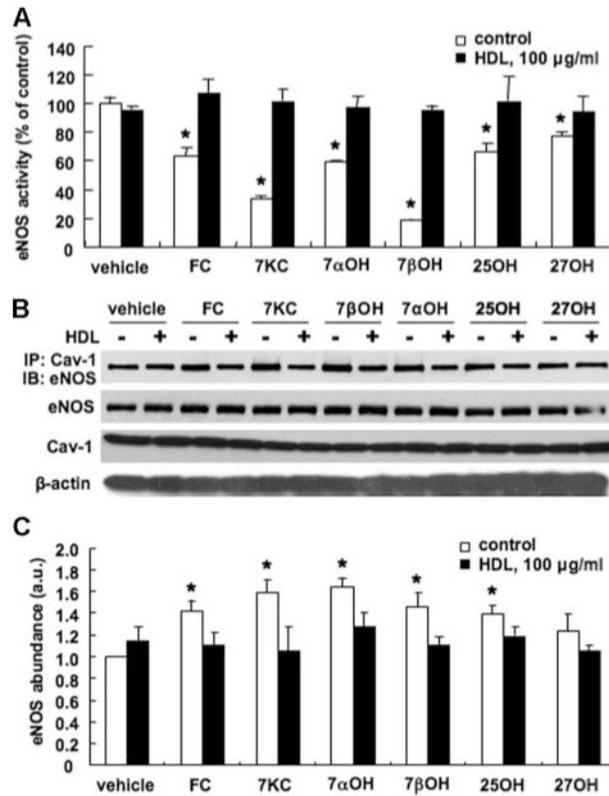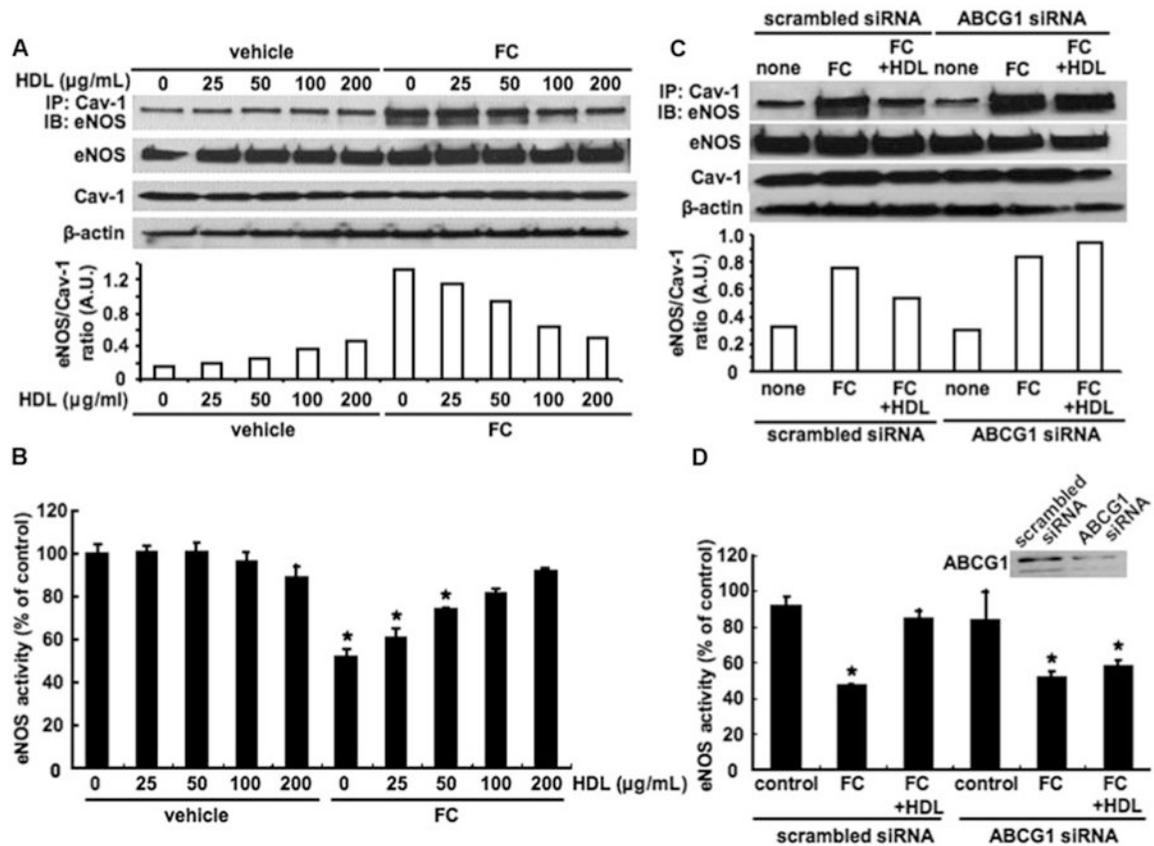
Figure 1.
Effects of cholesterol and different oxysterols on NOS activity and Cav-1/eNOS interaction in HAECs. The HAECs were incubated with cholesterol or different oxysterols (each 40 μg/mL, except for 7-ketocholesterol and 7β-hydroxycholesterol [10 μg/mL]) in the absence (control: open bars) or presence (HDL: filled bars) of HDL, 100 μg/mL, for 16 hours. Vehicle indicates no preincubation with cholesterol/oxysterols; FC, cholesterol; 7KC, 7-ketocholesterol; 7αOH, 7α-hydroxycholesterol; 7βOH, 7β-hydroxycholesterol; 25OH, 25-hydroxycholesterol; and 27OH, 27-hydroxycholesterol. A, eNOS activity. B, Western blot analysis of amount of eNOS coimmunoprecipitated (IP) with Cav-1, total eNOS, Cav-1, and β-actin. C, Quantification of eNOS IP with Cav-1. AU indicates arbitrary unit. The results are represented as mean±SEM of 3 individual experiments. *P<0.05 vs. vehicle control.

Figure 2.
Effects of HDL and ABCG1 expression on eNOS activity and Cav-1/eNOS interaction in
HAECs. A and B, The HAECs were incubated with cholesterol, 40 μg/mL, and HDL, 0 to
200 μg/mL, for 16 hours. C and D, The HAECs were transfected with scrambled or ABCG1
siRNA. Forty-eight hours after transfection, HAECs were incubated with cholesterol, 40
μg/mL, in the presence or absence of HDL, 100 μg/mL, for 16 hours. A and C, Western blot
analysis of amount of eNOS coimmunoprecipitated (IP) with Cav-1, total eNOS, Cav-1, and
β-actin. The ratio of eNOS in the caveolin-1 immunoprecipitate to total caveolin-1 is
quantified. A.U. indicates arbitrary unit. B and D, eNOS activity. The results are represented
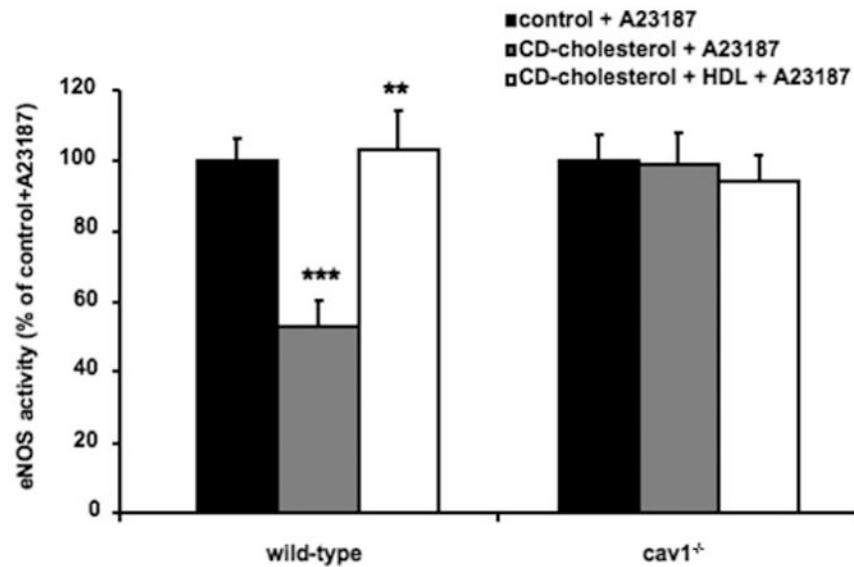as mean±SEM of 3 individual experiments. *$P<0.05$ vs vehicle control.

Figure 3.
Effect of Cav-1 on cholesterol- and HDL-mediated modulation of eNOS activity in MLECs. Wild-type and *cav1*$^{-/-}$ MLECs were incubated without (control) and with methyl-β-cyclodextrin (CD) cholesterol or with CD cholesterol (both 2-mmol/L cholesterol) and HDL, 100 μg/mL, for 3 hours. Then, cells were incubated with the Ca^{2+} ionophore (A23187) for 3 minutes and lysed; and eNOS activity was assessed on the lysate. *P<0.05 vs the wild-type control, and **P<0.05 vs the wild-type CD cholesterol.
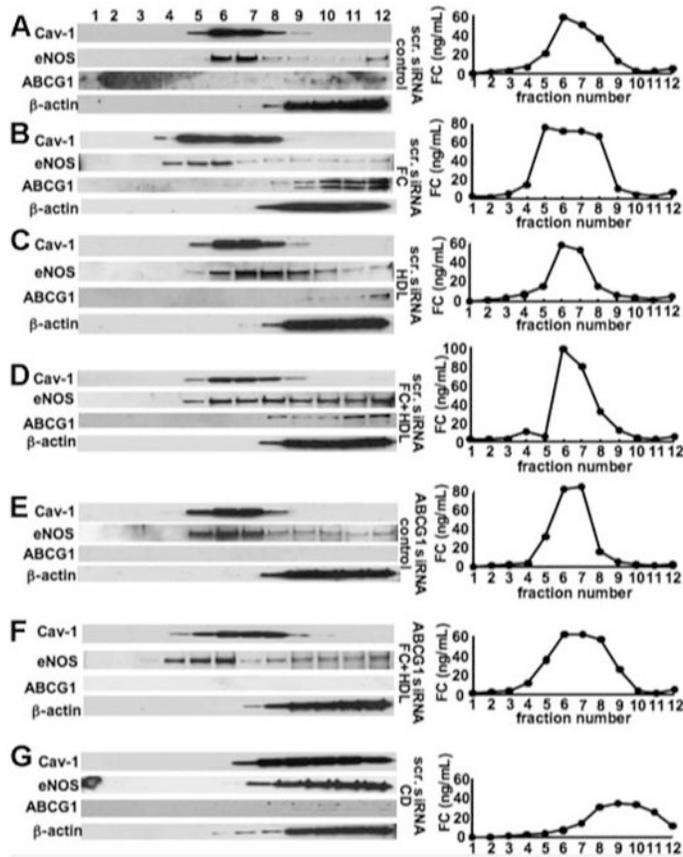
Figure 4.
Cav-1, eNOS, ABCG1, -actin, and cholesterol plasmalemmal distribution in HAECs. A–D,
The HAECs were transfected with scrambled siRNA (scr. siRNA). Forty-eight hours after
transfection, HAECs were incubated with medium alone (A); cholesterol, 40 g/mL (B);
HDL, 100 g/mL (C); or cholesterol + HDL (D) for 16 hours. E and F. The HAECs were
transfected with ABCG1 siRNA. Forty-eight hours after transfection, HAECs were
incubated with medium alone (E), or cholesterol, 40 g/mL, and HDL, 100 g/mL (F). G, The
HAECs were transfected with scrambled siRNA. Forty-eight hours after transfection,
HAECs were incubated with 10-mmol/L methyl-β-cyclodextrin (scr. siRNA, methyl-β-
cyclodextrin [CD]) for 1 hour. Western blotting for Cav-1, eNOS, ABCG1, and β-actin (left)
and cholesterol concentrations (right) from sucrose-gradient fractions isolated from HAECs
was performed as described. The results are represented from 1 experiment performed
independently 3 times. **$P<0.01$ vs the wild-type CD cholesterol; ***$P<0.001$ vs the wild
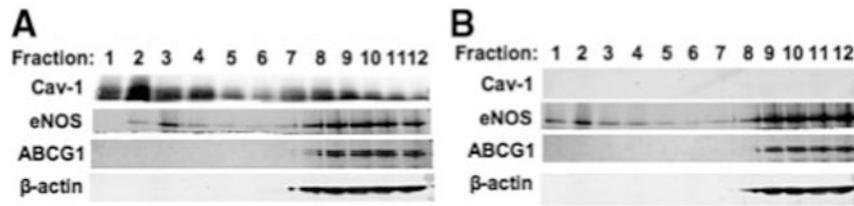type control.

Figure 5.
Cav-1, eNOS, ABCG1, and β-actin plasmalemmal distribution in MLECs. A and B, Western blot for Cav-1, eNOS, ABCG1, and β-actin from sucrose-gradient fractions isolated from wild-type MLECs (A) or Cav-1$^{-/-}$ MLECs (B) as described. The results are representative of 1 experiment performed 3 times.
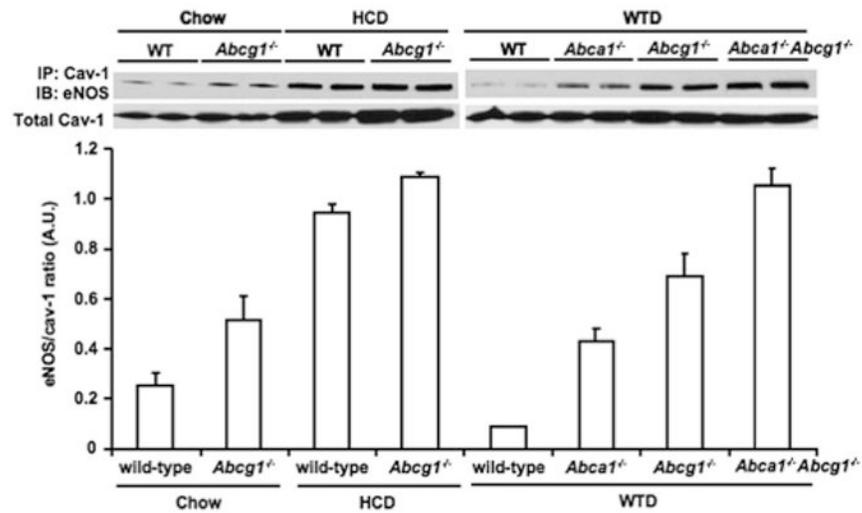
Figure 6.
Effects of ABC transporter deficiency and different diets on Cav-1/eNOS interaction in mouse aorta. Western blot analysis on amount of eNOS coimmunoprecipitated (IP) with Cav-1 (top) and Cav-1 in the total lysate (bottom). The HCD includes 1.25% cholesterol, 7.5% cocoa butter, and 0.5% sodium cholate; and the Western-type diet (WTD), 0.25% cholesterol and 21% milk fat. Chow indicates chow diet. Mice (n=4–5 in each group) were fed either diet for 11 to 12 weeks.[12] The immunoprecipations and Western blots were performed on 2 pools per mouse per genotype and represent 1 experiment that was performed 3 times. The ratio of eNOS in the caveolin-1 immunoprecipitate, after correction for total eNOS, to total caveolin-1 is quantified. A.U. indicates arbitrary unit; WT, wild type.