Hypercholesterolemia Promotes a CD36-dependent and Endothelial Nitric-oxide Synthase-mediated Vascular Dysfunction*

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Jeanie F. Kincer‡, Annette Uittenbogaard‡, James Dressman‡, Theresa M. Guerin‡, Maria Febbraio§, Ling Guo‡, and Eric J. Smart‡¶

From the ‡University of Kentucky Medical School, Department of Physiology, Lexington, Kentucky 40536 and §Department of Medicine, Division of Hematology-Medical Oncology, Weill Medical College of Cornell University, New York, New York 10021

Numerous studies have implicated either the presence or absence of CD36 in the development of hypertension. In addition, hypercholesterolemia is associated with the loss of nitric oxide-induced vasodilation and the subsequent increase in blood pressure. In the current study, we tested the hypothesis that diet-induced hypercholesterolemia promotes the disruption of agonist-stimulated nitric oxide generation and vasodilation in a CD36-dependent manner. To test this, C57Bl/6, apoE null, CD36 null, and apoE/CD36 null mice were maintained on chow or high fat diets. In contrast to apoE null mice fed a chow diet, apoE null mice fed a high fat diet did not respond to acetylcholine with a decrease in blood pressure. Caveolae isolated from in vitro vessels did not contain endothelial nitric-oxide synthase (eNOS).1 and activation of the enzyme. We have used an endothelial cell line to demonstrate that the depletion of caveola cholesterol will result in the re-localization of eNOS from caveolae to an internal membrane compartment, where it cannot be stimulated to demonstrate that the depletion of caveola cholesterol with extracellular agonists (15, 16).

**CD36 is a class B scavenger receptor that is expressed in cardiac myocytes, adipocytes, macrophages, platelets, and microvascular endothelial cells (1, 2). Recent studies with mice and rats that lack CD36 demonstrate a role for this protein in atherosclerosis (3), angiogenesis (4), and diabetes (5–7). Genetic linkage studies implicate the absence of CD36 in the development of hypertension (8), although direct experimental evidence is lacking. One of the confounding factors in the linkage studies is that genes involved in blood pressure regulation such as endothelial nitric-oxide synthase, leptin, and neuropeptide Y are linked to chromosome 4, making it difficult to attribute blood pressure to CD36 (8, 9).

Studies by Pravenec et al. (8) demonstrate that the transfer of a segment of chromosome 4 to spontaneous hypertensive rats (SHR) normalizes blood pressure; however, when just the low density lipoprotein fraction was transferred and did not normalize (10). We demonstrate that CD36 was transferred, suggesting that CD36 is involved. Because of the potential for hypertension to increase cholesterol levels, mice regaining responsiveness to acetylcholine with a decrease in blood pressure. Nitric oxide binds to soluble guanylate cyclase to produce cyclic G-nitro-L-arginine; TBS, Tris-buffered saline; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis( hydroxymethyl)ethyl]glycine; PVDF, polyvinylidene difluoride.

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†To whom correspondence should be addressed: University of Kentucky, Dept. of Physiology, 800 Rose St., MS 508 C, Lexington, KY 40536. Tel.: 859-323-6412; Fax: 859-323-1070; E-mail: ejsmart@uky.edu.
have a strong associative correlation (20, 21). The mechanism for this interaction is not known; however, recent studies with a heterologous in vitro system demonstrated that copper-oxidized LDL could deplete caveola cholesterol in a CD36-dependent manner, which resulted in the re-localization of eNOS and an inhibition of agonist-stimulated nitric oxide generation (15, 16). Furthermore, it was demonstrated that HDL could restore the cholesterol concentration of caveola, the localization of eNOS to caveola, and consequently the ability of agonists to stimulate eNOS. Although the in vitro data are suggestive of a mechanism that may link atherosclerosis and hypertension at the molecular level, data demonstrating that CD36 and caveola are involved in blood pressure regulation does not exist. To address this issue we used apoE null and apoE/CD36 null mice to determine whether CD36 is involved in blood pressure regulation. The data demonstrate that CD36 deficiency protected mice from hypercholesterolemia-induced vascular dysfunction. CD36 deficiency also protected caveola from cholesterol depletion, prevented nitric-oxide synthase re-localization, and maintained agonist-induced nitric oxide generation. In addition, experiments with isolated vessels demonstrated that the plasma LDL fraction is responsible for the reversible inhibition of agonist activation of eNOS. These studies provide the first molecular link between CD36 function, caveola integrity, nitric-oxide synthase activity, and blood pressure regulation. In addition, these data imply that endothelial CD36 may be a useful clinical target in the management of atherosclerosis-induced vascular dysfunction.

**EXPERIMENTAL PROCEDURES**

**Materials—**M199, BME vitamin mix, feta, trypsin-EDTA, OptiPrep, and penicillin/streptomycin were from In-vitrogen. The BME amino acid mix and the [3H]Arginine (specific activity, 5.11 Ci/mmol) were from Mingen. The anti-Gq IgG was from Santa Cruz, CA). Anti-mouse CD36 IgA was from Cascade Bioscience, MA). The GC G2579A system (Agilent, Palo Alto, CA) equipped with a SGE HT5 20 mm for femoral and sodium nitroprusside (1.0, 15.0, 30.0 μg/kg data are shown) and sodium nitroprusside (1.0, 15.0, 30.0 μg/kg; only the 1.0 μg/kg data are shown) and sodium nitroprusside (1.0, 15.0, 30.0 μg/kg; only the 1.0 μg/kg data are shown). Mice were continuously monitored during the procedure, and blood pressure was allowed to return to base line and then stabilized for ~30 s between doses. Catheters were flushed with heparin saline between drugs as well to prevent clot formation. Upon completion of data collection, mice were euthanized by CO2 administration.

**Cholesterol Mass Quantification—**Cholesteryl heptadecanoate was added to each vessel preparation to serve as an internal standard, and the samples were then extracted with isopropanol-hexane (22). The extracted lipid was derivatized by suspending the dried lipid in M-bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane/acetoni-trile (89:1:10). The material was heated at 80 °C for 5 min, dried, suspended in iso-octane, and used for gas chromatography (Protocol T496125B, Supelco, Sigma Aldrich). Authentic cholesterol was dissolved in iso-octane and used as a standard for the retention time of the mixture. The standards were spotted (splitless) onto an Agilent GC 6890 GC (2579A) system (Agilent, Palo Alto, CA) and connected to a SOE HT5 aluminum clad fused silica capillary column (12 m × 0.32 mm × 0.1 μm, Supelco, Bellefonte, PA), after 400 °C (10 °C/min) and held for 3.5 min. A model 5973 mass-selective detector (Agilent Technologies) was used in both scan and selected ion-monitoring modes to identify the samples.
Radiolabeled Cholesterol Determination—Thin-layer chromatography and liquid scintillation counting was used to measure the amount of \[^{3}H\]sterol in each sample (23). Each sample was adjusted to a volume of 1 ml with distilled water. Methanol (1.2 ml) containing 2% (v/v) acetic acid was added to the sample before vortexing 2 times, 30 s each. Chloroform and methanol were then added, and the sample vortexed 2 times, 30 s each. The organic and aqueous phases were separated in a Beckman clinical centrifuge at 3750 rpm, 15 min, 25 °C. The organic phase was dried under nitrogen and then isolated in 50 µl of the solvent system (80:20:1; petroleum ether/ethyl ether/acetic acid). Pure cholesterol was dissolved in the solvent system and used as a standard (5 µg/µl). Lipids were visualized by spraying with sulfuric acid-ethanol and heating at 180 °C for 10 min. Unlabeled cholesterol was added to each fraction to facilitate visualization. The appropriate spots were scraped, and the amount of radiation was quantified by liquid scintillation counting.

Caveolae Isolation—Caveolae were isolated from mouse endothelium as described by Schnitzer and et al. (24–26) with the modifications described below. A mouse was anesthetized with ketamine/xylazine (see above), then a vertical incision was made into the abdomen. A catheter was placed in the isolated descending aorta just distal to the renal artery and vein. To allow the perfusion to flow into the abdominal cavity, a small incision was made with scissors into the caudal vena cava. The perfusate went into the aorta, through the femoral arteries and capillaries of each leg, and then returned to the abdomen through the femoral vein and the caudal vena cava.

An infusion pump was used to perfuse 10 ml of each of the following solutions (2 ml/min): 1) 0.2 µg/ml nitroprusside in Ringers (Sigma K-4002) at room temperature, 2) 0.2 µg/ml nitroprusside in Ringers at 10 °C, 3) MES-buffered saline (MBS; 135 mM NaCl, 20 mM MES, pH 6.0, 10 °C, 4) 1% silica (TOPOGEN positively charged colloidal silica, Columbus, Ohio) in MBS, 10 °C, 5) MBS, 10 °C, 6) 0.1% polyacrylamide (Polyscience, Inc. #00627) in MBS, 10 °C, 7) Sucrose/KCl/HEPES, mm sucrose, 20 mM KCl, 25 mM HEPES, pH 7.4, 10 °C. After the femoral arteries from both hind limbs were collected, placed on an iced aluminum block, and covered with 10 µg/ml protease inhibitors and centrifuged at 38,580 × g for 5 min, the endothelial plasma membrane-bead complexes were separated in a 12.5% SDS-polyacrylamide gel using the method of Laemmli (30). The separated proteins were then transferred to PVDF. The PVDF was blocked in TBS that contained 5% dry milk for 1 h at room temperature. Primary antibodies were diluted in TBS that contained 1% dry milk and incubated with the PVDF for 1 h at room temperature. The PVDF was washed 4 times, 10 min each in TBS plus 1% dry milk. The secondary antibodies (all conjugated to horseradish peroxidase) were diluted 1:20,000 in TBS plus 1% dry milk and incubated with the PVDF for 1 h at room temperature. The PVDF was then washed, and the bands were visualized by chemiluminescence.

Nitric-oxide Synthase Assay—NOS activation was determined in intact cells or vessels as described (31, 32). Briefly, the cells were plated into 12-well plates at 5,000 cells/well and grown to 60% confluency, or one vessel was placed per well. The cells were treated as described under “Results” and the measured product of the mmyastic activity, eNOS initiated by adding 0.75 µM l-[\(^{3}H\)arginine/well. The NOS reaction was terminated by adding 500 µl of ice-cold 1 N trichloroacetic acid to each well. The cells were freeze-fractured twice in liquid nitrogen for 2 min with thawing at 37 °C for 5 min and then scraped with a rubber spatula. The contents of each well were then transferred to ice-cold glass test tubes. Ether extraction was performed 3 times with water-saturated ether to remove the trichloroacetic acid. The samples were neutralized with 1.5 ml of 25 mM HEPES, pH 8, applied to Dowex AG50WX-8 (Tris form) columns, and eluted with 1 ml of 40 mM HEPES buffer, pH 5.5, containing 2 mM EDTA and 2 mM EGTA. l-[\(^{3}H\]Citrulline was collected in scintillation vials and quantified by liquid scintillation counting. In individual experiments performed in 12-well plates, 4 wells were used for each treatment group. Findings were confirmed in at least 3 independent experiments. NOS activation in the intact cells was completely inhibited by 2 mM l-NNA.

RESULTS

Acetylcholine-induced Vasodilation—To test the hypothesis that CD36-deficient mice protected from hypercholesterolemia-induced vascular dysfunction, we used age and sex-matched mice fed a high fat diet for 6 weeks. The animals used in these studies were in a C57BL/6 background strain, apoE null, CD36 null, and apoE/CD36 null. To study the animals were fed a high fat diet and cholesterol-induced decrease in blood pressure of 26 ± 8 mmHg in all of the groups, indicating that the vessels were capable of responding to acetylcholine. As expected, the nitric-oxide synthase inhibitor, l-NNA, did not inhibit the response to sodium nitroprusside because sodium nitroprusside chemically breaks down to nitric oxide independent of NOS enzymatic activity. All of the chow-fed animals and C57BL/6 and CD36 null mice fed a high fat diet had a transient decrease in blood pressure of 24 ± 9 mmHg after infusion with 1 µg/kg of acetylcholine (Fig. 1, A and B). In striking contrast, apoE null mice fed a high fat diet did not respond to acetylcholine infusion with a decrease in blood pressure (Fig. 1B). Importantly, apoE/CD36 null mice fed a high fat diet responded to acetylcholine with a transient decrease in blood pressure similar to control animals (Fig. 1B). Infusion of l-NNA completely inhibited the acetylcholine-induced decrease in blood pressure. Similar data were obtained when 1 µg/kg bradykinin was used to induce vasodilation (data not shown).

eNOS Re-localization—We next began to dissect the molecular mechanism responsible for 1) the lack of a response to acetylcholine in apoE null mice fed a high fat diet and 2) the protective effect afforded by CD36 deficiency. The data presented above indicated that acetylcholine infusion induced vasodilation in a NOS-dependent manner. Thus, one possible explanation could be the loss of eNOS in high-fat-fed apoE null mice but not in high-fat-fed apoE/CD36 null mice. To test this possibility femoral artery vessels were isolated from similar groups of animals as those used for Fig. 1 and processed to generate protein lysates. The lysates were then resolved by
SDS-PAGE and immunoblotted with eNOS IgG. Because eNOS is not expressed in other vascular cells, the immunoblot signal can be associated with endothelial cells. Both apoE null mice and apoE/CD36 null mice fed a chow or high fat diet contained similar amounts of eNOS in the whole vessel (Fig. 2A, Whole Vessel). Because caveolae can influence eNOS activity (19) and be- cause the total immunodetectable eNOS in the vessel was not altered, we next determined if the subcellular localization of eNOS changed. To determine whether eNOS was localized to endothelial caveolae in apoE null and apoE/CD36 null mice fed a high fat diet, we used a published method to isolate caveolae in vivo (24, 25). In brief, silica beads were perfused through the femoral artery and then cross-linked before extracting the vessel. Endothelial plasma membranes were then isolated, and caveolae were obtained by differential density gradient centrifugation followed by immunoisolation with caveolin IgG (26, 29, 31). The proteins associated with caveolae were resolved by SDS-PAGE and immunoblotted with eNOS regardless of being fed a chow or high fat diet. Preliminary studies with different concentrations of sodium nitroprusside and acetylcholine demonstrated that the amounts used were sufficient for a maximal response (data not shown). Results reflect the mean ± S.E., n = 11–15; *, p < 0.01 with respect to C57BL/6 in each group.

The data suggested that the inability of acetylcholine to induce vasodilation in apoE null mice fed a high fat diet was due to the mis-localization of eNOS, it was also possible that some other component of the signaling pathway was missing. To address this possibility we also examined the total vessel levels and caveola levels of CD36 (Fig. 2C), acetylcholine receptors (Fig. 2D), and hetero-trimeric G protein (Gq) (Fig. 2E). Fig. 2 demonstrates that CD36 was present in apoE null mice but not apoE/CD36 null mice, whereas acetylcholine receptors and Gq were enriched in caveolae isolated from all of the animals. Numerous groups have reported that the in vitro and phar-
macological depletion of caveola cholesterol results in the disruption of caveola-mediated signal transduction (34–37). However, it has never been demonstrated that depletion of caveola cholesterol is involved in eNOS-mediated signal transduction in a physiologically relevant model of disease. To determine whether a decrease in caveola cholesterol affects the movement of eNOS out of caveola, we used the silica bead method (24, 25) followed by isolating femoral arteries from apoE null and CD36 null mice fed a high fat diet. Caveolae isolated from apoE null (Fig. 4A) or apoE/CD36 null (Fig. 4B) high fat plasma were unable to produce nitric oxide in response to acetylcholine; however, apoE null or apoE/CD36 null high fat plasma did not contain eNOS. Caveolae isolated from chow-fed apoE null vessels were depleted of cholesterol (Fig. 3). Caveolae isolated from chow-fed apoE null vessels contained similar amounts of eNOS regardless of localization to caveola. 20 μg of eNOS were isolated from each group of animals and resolved by SDS-PAGE and immunoblotted with eNOS IgG. Because equal amounts of proteins were loaded for each lane, the greater intensity of bands in the caveolae lanes indicates that the protein is contained within the caveolae in vivo. In contrast, when apoE null high fat vessels were incubated with apoE null high fat plasma, eNOS no longer associated with caveolae. Caveolae isolated from cholesterol-enriched vessels maintained the ability to maximally stimulate eNOS in response to acetylcholine (Fig. 5). The maximal eNOS response was determined by incubating the appropriate vessels in 2 μg/ml of ionomycin, a calcium ionophore. ApoE null chow vessels maintained in apoE null or apoE/CD36 null chow plasma maintained the ability to maximally stimulate eNOS in response to acetylcholine (Fig. 5). The maximal eNOS response was determined by incubating the appropriate vessels. However, apoE null high fat vessels maintained in apoE null or apoE/CD36 null high fat plasma did not contain eNOS. Importantly, apoE null vessels were unable to produce nitric oxide in response to acetylcholine (Fig. 5). The maximal eNOS response was determined by incubating the appropriate vessels in 2 μg/ml of ionomycin, a calcium ionophore. ApoE null chow vessels maintained in apoE null or apoE/CD36 null chow plasma maintained the ability to maximally stimulate eNOS in response to acetylcholine. However, similar vessels maintained in high fat plasma did not contain functional eNOS. ApoE null high fat vessels maintained in apoE null or apoE/CD36 null chow plasma were unable to produce nitric oxide in response to acetylcholine. In contrast, when apoE null high fat vessels were placed in apoE null or apoE/CD36 null chow plasma, the ability to respond to acetylcholine was restored. Importantly, all of the vessels responded similarly to ionomycin, which indicated that the vessels contain functional eNOS even if agonists did not activate the enzyme.

We next determined if the amount of cholesterol associated with caveoleae could be altered by incubating vessels in chow or high fat plasma (Fig. 6). The amount of cholesterol associated with caveolae isolated from apoE/CD36 null vessels was not affected by any of the treatments. In contrast, caveolae in vessels isolated from chow-fed apoE null mice were depleted of cholesterol after incubation in apoE null (Fig. 6A) or apoE/
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Fig. 5. eNOS activity in isolated vessels incubated with plasma. ApoE and CD36/apoE mice were fed a high fat diet for 6 weeks and used to isolate plasma. Vessels isolated from either chow-fed or high fat-fed animals were incubated with chow or high fat plasma for 16 h. Acetylcholine-stimulated eNOS activity was measured in the presence or absence of ionomycin. Ionomycin-stimulated eNOS activity was measured in the presence or absence of plasma. The data presented thus far suggest that the removal of plasma component(s) promotes the inhibition of eNOS activity, which is capable of depleting caveolae of cholesterol. The LDL isolated from apoE null mice were incubated in plasma containing [3H]acetate to label the radiolabeled cholesterol, whereas the chow LDL did not contain significant amounts of radiolabeled cholesterol. The cells were washed and incubated for 16 h in LPDP only or LPDP plus HDL, LDL, or VLDL (10 μg/ml) from chow-fed or high fat-fed apoE null mice. After the incubation, the medium containing the lipoproteins were collected, the lipids were extracted, and cholesterol was separated by thin-layer chromatography and quantified by liquid scintillation spectroscopy. High fat LDL contained a large amount of radiolabeled cholesterol, whereas the chow LDL did not contain significant amounts of radiolabeled cholesterol (Fig. 8). The HDL, VLDL, and LPDP fractions from either chow or high fat-fed mice did not contain significant amounts of radiolabeled cholesterol.

Cyclodextrin Mimics the Effects of High Fat Plasma/LDL—The data suggest that the LDL in high fat-fed mice deplete caveolae of cholesterol in a CD36-dependent manner and that this depletion of cholesterol promotes the re-localization and subsequent inhibition of eNOS stimulation. To further validate this conclusion we used an independent method to deplete caveolae of cholesterol in intact vessels. We treated vessels isolated from chow-fed apoE null mice with 5 mM cyclodextrin for 2 h and then measured the amount of cholesterol associated with caveolae (Fig. 9A) and agonist-stimulated eNOS activity (Fig. 9B). Cyclodextrin depletes cells of cholesterol and has been widely used as a reagent to disrupt the function of caveolae...
lae (34–38). Cyclodextrin treatment depleted caveolae of cholesterol to the same extent as high fat plasma (Fig. 9A). In addition, the cyclodextrin-mediated depletion of caveolae cholesterol correlated with an inability of acetylcholine to stimulate eNOS activity (Fig. 9B).

**DISCUSSION**

Genetic linkage studies and *in vitro* studies suggest that CD36 may be involved in the development of hypertension; however, the direct involvement of CD36 has not been demonstrated (6,8–10,41). In the present studies, we used CD36 null mice and apoE/CD36 null mice to determine whether 1) the absence of CD36 affected blood pressure or blood pressure regulation and 2) if the absence of CD36 in a hypercholesterolemic environment affected blood pressure or blood pressure regulation. The data demonstrate that the absence of CD36 in mice fed a chow or high fat diet does not affect resting blood pressure or the ability to respond to physiological vasodilatory agonists such as acetylcholine and bradykinin. CD36 null mice have similar levels of serum cholesterol as C57BL/6 mice. In contrast, apoE null mice and apoE/CD36 null mice had slightly evaluated serum cholesterol levels when fed a chow diet, and the animals became hypercholesterolemic when fed a high fat diet. In this pro-cardiovascular disease environment apoE null mice lost the ability to vasodilate in response to acetylcholine, whereas apoE/CD36 null mice still responded to acetylcholine. These data demonstrate that the absence of CD36 protects against an eNOS-dependent vascular dysfunction, or stated...
differently, in a hypercholesterolemic environment, CD36 promotes an eNOS-mediated vascular dysfunction. The in vivo data clearly demonstrate that CD36 mediates an eNOS-dependent vascular dysfunction in a hypercholesterolemic environment. Earlier work by our laboratory demonstrated that copper-oxidized LDL can efflux cholesterol from caveola and promote the translocation of eNOS out of caveola (15, 16). Extracellular agonists could not stimulate eNOS once the enzyme was no longer associated with caveola (15, 16). A major limitation with these earlier studies is that copper-oxidized LDL is not a physiological ligand, and the observed cholesterol efflux may have been the result of an in vitro artifact. By using an in vitro method to isolate endothelial plasma membranes, we now demonstrate that a high fat diet in apoE null mice promotes the translocation of eNOS away from caveola, whereas apoE/CD36 null mice fed the same high fat diet retained eNOS in caveola. Importantly, the diets did not affect the overall level of eNOS in the vessel but only the subcellular location of the enzyme. In addition, other possible explanations for the lack of a response to acetylcholine were ruled out, such as the level of acetylcholine receptors and hetero-trimeric G proteins. Overall, the data suggest that the re-localization of eNOS was responsible for the lack of agonist-induced eNOS activity.

Numerous factors can influence the subcellular localization of eNOS including, acylation, phosphorylation, interaction with caveolin, and caveola cholesterol levels (20, 42, 43). Endothelin-nitric oxide synthase has been reported to be associated with both caveola and the Golgi (44). The relationship of caveola-localized eNOS and Golgi-localized eNOS is unclear. However, recent data suggest that caveola begin to assemble in the Golgi (45-47) and that Golgi-localized eNOS is fact associated with nascent caveola. The phosphorylation of eNOS by agonists has been shown to translocate to the cytosol, and the Golgi is responsible for controlling the amount of eNOS present in the Golgi. The state of eNOS is not completely understood (19, 48). The phosphorylation of eNOS by agonists has been reported to be associated with caveolae (15). Consistent with the earlier studies, we did not detect phosphorylation of eNOS by using commercially available phospho-eNOS-specific antibodies (data not shown). However, the in vivo data demonstrated that, in endothelial cells, CD36 mediates the efflux of caveola cholesterol to copper-oxidized LDL. The depletion of caveola cholesterol caused eNOS to translocate to an unidentified intracellular compartment where it could not be stimulated by agonists (15). In the current study, we demonstrated for the first time that apoE null mice fed a high fat diet lost an enrichment of cholesterol in the isolated caveola fraction. However, caveola isolated from apoE/CD36 null mice fed a high fat diet were highly enriched in cholesterol. These data suggest that the absence of CD36 protects mice from a vascular dysfunction by protecting caveola from the loss of cholesterol. The ability of CD36 to efflux cholesterol from endothelial cells is unique because CD36 in macrophage is generally thought of as a mechanism for the net uptake of sterol. However, endothelial cells are net exporters of cholesterol and do not accumulate large amounts of sterol, whereas macrophages have the capability to internalize and store sterols (51). The molecular mechanism of how CD36 mediates the efflux of caveola cholesterol is unclear; however, other investigators demonstrate that the protein caveolin is required for the net efflux of cholesterol from caveola to extracellular acceptors (52, 53). It is also important to note that CD36 only mediated an efflux of caveola cholesterol in mice that were fed a high fat diet. The data implicated a plasma component in the hypercholesterolemic mice as the causative factor for the vascular dysfunction. If this was the case then we reasoned that plasma isolated from high fat-fed mice should be able to inhibit agonist-stimulated eNOS activity in a vessel isolated from a chow-fed apoE null mouse. Furthermore, we wanted to determine whether it was possible to restore a dysfunctional vessel to a "normal" vessel; therefore, we incubated high fat vessels with plasma isolated from chow-fed mice. High fat plasma isolated from apoE null or apoE/CD36 null mice when added to vessels isolated from chow-fed mice promoted the loss of eNOS from caveola, inhibition of agonist-stimulated eNOS, and depletion of caveola cholesterol. Importantly, the incubation of vessels isolated from high fat-fed mice (dysfunctional) with chow plasma resulted in a return of eNOS to caveola, a return of NOS activity, and a return of caveola cholesterol. Fractionation of the plasma demonstrated that only the LDL fraction from high fat-fed animals has the capability of inhibiting eNOS stimulation and depletion of caveola cholesterol. These data illustrate several important findings. The fact that LDL from both apoE null and apoE/CD36 null mice caused the dysfunction indicate that apoE is not required, due to some difference in the lipoprotein profile of mice. Second, the data demonstrate that depletion of caveola cholesterol is responsible for the re-localization of eNOS. The current study is the first demonstration that CD36 plays a direct role in the development of an eNOS-mediated vascular dysfunction. The data also suggest that the mechanism of CD36-mediated atherosclerotic dysregulation is the disruption of caveola and de-acylation of eNOS. The depletion of caveola cholesterol away from caveola. Another important finding is that eNOS dysregulation is reversible; that is, when hypercholesterolemic plasma is replaced with normal plasma, the dysfunctional vessels can regain function. These findings suggest that lowering plasma cholesterol levels in patients may not only effect atherosclerotic lesions but also have direct effects on endothelium vasoreactivity. In addition, the demonstration of CD36 involvement in this process provides a potentially useful therapeutic target for maintaining vascular reactivity in hypercholesterolemic patients.

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REFERENCES
1. Silverstein, R. L., and Feinten, M. (2000) Curr. Opin. Lipidol. 11, 483–491
2. Fehrman, M., Hajjar, D. P., and Silverstein, R. L. (2001) J. Clin. Invest. 108, 785–791
3. Fehrman, M., Podzél, A. E., Smith, J. D., Hajjar, D. P., Hagen, S. L., Hoff, H. P., Sharma, K., and Silverstein, R. L. (2000) J. Clin. Invest. 105, 1049–1056
4. Dawson, D. W., Pearce, S. F., Zhong, R., Silverstein, R. L., Frazier, W. A., and Bouché, P. P. (1997) J. Biol. Cell Biol. 138, 707–717
5. Aitman, T. J. (2001) Lancet 357, 654–655
6. Aitman, T. J., Glazier, A. M., Wallace, C. A., Cooper, L. D., Norrisworthy, J. P., Wahid, F. N., Al-Majali, K. M., Tremblay, F. M., Mann, C. J., Shoul, C. C., Graf, D., Stolz, E., Kutz, T. W., Kroun, V., Pronovost, M., Braham,
