Lipids: potential regulators of nitric oxide generation

Gentle Chikani, Weifei Zhu, and Eric J. Smart
Department of Pediatrics, University of Kentucky Medical School, Lexington, Kentucky 40536-0230

CAVEOLAE AND NITRIC OXIDE

Endothelial nitric oxide synthase (eNOS) is an enzyme that generates nitric oxide in the endothelium (15, 20, 27, 33, 35). Nitric oxide is a labile and a potentially cytotoxic molecule, and the location of nitric oxide generation is critical to its ultimate function (35). A significant amount of eNOS is found in specialized lipid rafts called caveolae. Caveolae contain proportionally small amounts of phospholipids and large amounts of cholesterol, sphingomyelin, and glycosphingolipids. Caveolae also contain the class B scavenger receptors SR-BI and CD36, which interact with lipoproteins and affect eNOS function (see below) (2, 32). One of the earliest studies to demonstrate a link between eNOS function and lipids showed that acylation of eNOS targets the protein to caveolae (38). Myristoylation of the NH2-terminal glycine residue in eNOS and palmitoylation of the cysteine residues in positions 15 and 26 target eNOS to caveolae. The importance of this localization was further established when it was shown that the caveolae protein, caveolin, can function as a negative regulator of eNOS (14, 17). In addition, it has been demonstrated that receptor-mediated stimulation of eNOS with acetylcholine or bradykinin requires that eNOS be localized in caveolae (3, 38). Surprisingly, studies have not been conducted to determine whether different dietary fatty acids can alter the acylation of eNOS and/or the targeting of eNOS to caveolae.

OXIDIZED LDL, CAVEOLAE CHOLESTEROL, AND eNOS FUNCTION

Hypercholesterolemia-induced vascular diseases are characterized by an early and selective attenuated responsiveness to receptor-dependent stimuli of endothelium-derived relaxation, in particular, nitric oxide. Feron et al. (13) provided a key mechanistic link between hypercholesterolemia and eNOS function. Using a bovine aortic endothelial cell culture system, they demonstrated that hypercholesterolemic serum, but not normal cholesterolemic serum, induced a dramatic upregulation of caveolin. The increase in cellular caveolin promoted an increase in the inhibitory caveolin-eNOS heterocomplex. Other studies using the atherogenic lipoprotein oxidized LDL (ox-LDL) demonstrated that oxLDL can induce the translocation of caveolin and eNOS away from caveolae to an internal membrane compartment, where the enzyme cannot be stimulated with agonists (3, 40). The oxLDL studies did not report an increase in the level of caveolin, suggesting that a component in hypercholesterolemic serum other than oxLDL is responsible for the increase in caveolin levels. This component has not been identified.

Further mechanistic studies revealed novel interactions between lipoproteins, lipoprotein receptors, caveolae, and eNOS. Metabolic labeling experiments showed that oxLDL caused an efflux of caveolae cholesterol out of the cell and onto the oxLDL (3). The lack of cholesterol in caveolae prevented a stable interaction between caveolae and the acyl groups on eNOS, which then resulted in the translocation of eNOS and caveolin to an internal membrane (3, 16). Depletion of caveolae cholesterol with cyclodextrin also caused a redistribution of both eNOS and caveolin to an intracellular membrane (3, 16). The effects of oxLDL on caveolae cholesterol and, consequently, nitric oxide generation required the presence of CD36 (40). CD36 is a class B scavenger receptor that binds to several ligands, including oxLDL (1, 37). Importantly, the link be-
tween CD36 and nitric oxide regulation of blood pressure was demonstrated by using apoE null and apoE/CD36 null mouse models (29). ApoE null mice are a commonly used model for atherosclerosis studies. ApoE is the major protein component of LDL particles, and the absence of this protein promotes an increase in plasma cholesterol levels that subsequently promotes the development of atherosclerosis. The severity of the atherosclerosis can be increased by feeding the mice a high-fat diet (11). Diet-induced hypercholesterolemia inhibited the ability of acetylcholine to induce vasodilation in apoE null mice; however, the lack of CD36 protected apoE/CD36 null mice from the same inhibition. Furthermore, the absence of CD36 protected caveolae from cholesterol depletion and the translocation of eNOS out of caveolae, thereby maintaining the ability of acetylcholine to stimulate nitric oxide production. It is unclear how the translocation of eNOS and caveolin out of caveolae prevents eNOS stimulation. One possibility is that the proteins are sequestered inside an internal membrane, where the enzyme is inaccessible to necessary cofactors such as tetrahydrobiopterin.

### HDL, CAVEOLAE CHOLESTEROL, AND eNOS FUNCTION

HDL can lower the risk of cardiovascular disease by several complex mechanisms that are incompletely understood (4, 19, 21). Several recent studies have illustrated that HDL can stimulate production of nitric oxide, which may contribute to the positive cardiovascular effects of HDL. Yuhanna et al. (42) demonstrated that HDL binding to its receptor, a class B scavenger protein called SR-BI, caused the activation of eNOS, with the resultant generation of nitric oxide. Additional studies demonstrated that HDL binding to SR-BI induces the activation of Akt kinase, which subsequently phosphorylates eNOS on Ser1179 and stimulates the enzyme to synthesize nitric oxide (34). In contrast, studies by Li et al. (31) showed that SR-BI-HDL interaction stimulated eNOS activity independently of Akt kinase and was mediated by a ceramide-dependent pathway. Ceramide is generated by the metabolism of sphingomyelin to sphingosine and ceramide (25). Caveolae are enriched in sphingomyelin, and caveolae-localized sphingomyelin may serve as the substrate for the generation of the ceramide that stimulates eNOS. The stimulation of eNOS by ceramide was reported previously by Igarashi et al. (26). Although the molecular mechanism by which ceramide promotes eNOS activity has not been elucidated, it is another intriguing example of lipid regulation of this critical enzyme. In addition, the apparent discrepancy between the Akt kinase-dependent activation and the ceramide-dependent activation has not been resolved. One possibility is that endothelial cells from different vascular beds exhibit distinct molecular responses under specific physiological conditions.

In addition to the direct effects of HDL and SR-BI on eNOS activation, other studies (3, 40) have shown that HDL and SR-BI can positively influence eNOS function, and presumably cardiovascular disease, by counteracting the oxLDL-initiated CD36-dependent depletion of caveolae cholesterol. As oxLDL and CD36 remove cholesterol from caveolae, HDL and SR-BI move cholesterol into caveolae. The net result is that the cholesterol level of caveolae is maintained, which allows...
eNOS to remain associated with caveolae. The above studies (3, 40) were conducted using cultured endothelial cells; thus it remains to be established whether oxLDL/CD36 and HDL/SR-BI counteract one another in this manner in vivo. It is interesting to speculate that, as plasma HDL levels drop and plasma LDL levels rise, a two-step dysfunction occurs. Initially the decrease in HDL may be seen physiologically only as an inability of HDL to stimulate eNOS, whereas another agonist such as acetylcholine may still trigger a response. However, as cardiovascular diseases progress and levels of LDL and oxLDL increase, a more global disruption of eNOS function may occur with the alteration of caveolae cholesterol levels. Once caveolae cholesterol levels are decreased, eNOS agonists would not stimulate nitric oxide production. Clearly, these questions require additional studies before they will be answered.

**ESTRADIOL AND eNOS ACTIVITY**

The role of estradiol, a steroid/lipid hormone, in cardiovascular disease is controversial; however, several well-controlled studies have demonstrated that estradiol can stimulate the production of nitric oxide (5, 22, 23, 28, 30, 39). Chambliss and Shaul (7) demonstrated that eNOS is stimulated by estradiol via nongenomic actions (independent of nuclear events) of estrogen receptor-α. Treatment of cells with estradiol caused an increase in eNOS activity in isolated endothelial cell plasma membranes, which was blocked by incubation with an estrogen receptor antagonist, ICI-182780, or incubation with an antibody against estrogen receptor-α. Estradiol also stimulates eNOS activity in COS-7 cells that expressed both eNOS and estrogen receptor-α, but not in cells that expressed only eNOS or only estrogen receptor-α. Further experiments demonstrated that estrogen receptor-α was associated with caveolae and that estradiol stimulates eNOS in a caveolae-dependent and an estrogen receptor-α-dependent manner. The authors concluded that estrogen receptor-α localized to endothelial cell caveolae and was coupled in a functional signaling module with eNOS and calcium (8, 10).

Recently an exciting link was established between HDL, estradiol, SR-BI, and the generation of nitric oxide (8–10, 18, 24, 31, 42). Gong et al. (18) demonstrated that HDL isolated from females (mice or humans) contained a significant amount of estradiol, whereas HDL isolated from males did not contain estradiol. The female HDL stimulated eNOS to produce nitric oxide, whereas the male HDL did not stimulate nitric oxide production. The effects of HDL on nitric oxide production were tested by using a human microvascular endothelial cell line and measuring the conversion of radiolabeled arginine to radiolabeled citrulline (18). Because one molecule of citrulline is generated for one molecule of nitric oxide, the amount of nitric oxide produced can be determined. Experiments with isolated vessels showed that female HDL induced relaxation in an eNOS-dependent manner, whereas male HDL did not induce any relaxation. Similar experiments using arteries isolated from SR-BI null mice clearly demonstrated that the ability of female HDL to induce relaxation required the presence of SR-BI. Importantly, these studies were extended to humans by examining this effect with HDL isolated from premenopausal women, postmenopausal women, and postmenopausal women receiving estrogen replacement therapy. Consistent with the other experiments, HDL isolated from both premenopausal women and from postmenopausal women receiving estrogen replacement therapy stimulated eNOS, whereas the HDL isolated from postmenopausal women did not. These studies revealed a novel mechanism linking plasma HDL levels, estradiol, and SR-BI to the generation of nitric oxide. Previous studies that examined the ability of HDL to stimulate eNOS did not distinguish between male and female HDL. Thus it is unclear whether only female HDL, and thus estrogen, can stimulate eNOS.

**SUMMARY**

The activity of eNOS and the consequent generation of nitric oxide are influenced by several lipids. It is well established that acylation of eNOS can directly affect the localization and therefore the function of the enzyme. Less well understood but potentially of great clinical significance is the link between oxLDL, CD36, HDL, and SR-BI and eNOS activity. It appears that these factors can influence eNOS activity indirectly by altering the cholesterol content of caveolae or directly by initiating a signaling cascade. The nongenomic effects of estrogen on eNOS activity are another link between plasma levels of lipids and nitric oxide that requires additional study. Clearly additional studies are required to unravel the overlapping mechanistic control of eNOS.

**REFERENCES**


**GRANTS**

This work was supported in part by grants from the National Heart, Lung, and Blood Institute (HL-64056, HL-62844, and HL-68509 to E. J. Smart) and the National Center for Research Resources at National Institutes of Health (P20-RR-15592). We are especially thankful for support provided by the Barnstable-Brown Endowed Chair.