Plasma HDL levels are inversely related to the incidence of atherosclerotic disease. Some of the atheroprotective effects of HDL are likely mediated via preservation of EC function. Whether the beneficial effects of HDL on ECs depend on its involvement in cholesterol efflux via the ATP-binding cassette transporters ABCA1 and ABCG1, which promote efflux of cholesterol and oxysterols from macrophages, has not been investigated. To address this, we assessed endothelial function in $\text{Abca1}^{-/-}$, $\text{Abcg1}^{-/-}$, and $\text{Abca1}^{-/-}\text{Abcg1}^{-/-}$ mice fed either a high-cholesterol diet (HCD) or a Western diet (WTD). Non-atherosclerotic arteries from WTD-fed $\text{Abcg1}^{-/-}$ and $\text{Abca1}^{-/-}\text{Abcg1}^{-/-}$ mice exhibited a marked decrease in endothelium-dependent vasorelaxation, while $\text{Abca1}^{-/-}$ mice had a milder defect. In addition, eNOS activity was reduced in aortic homogenates generated from $\text{Abcg1}^{-/-}$ mice fed either a HCD or a WTD, and this correlated with decreased levels of the active dimeric form of eNOS. More detailed analysis indicated that ABCG1 was expressed primarily in ECs, and that these cells accumulated the oxysterol 7-ketocholesterol (7-KC) when $\text{Abcg1}^{-/-}$ mice were fed a WTD. Consistent with these data, ABCG1 had a major role in promoting efflux of cholesterol and 7-KC in cultured human aortic ECs (HAECs). Furthermore, HDL treatment of HAECs prevented 7-KC–induced ROS production and active eNOS dimer disruption in an ABCG1-dependent manner. Our data suggest that ABCG1 and HDL maintain EC function in […]
ABCG1 and HDL protect against endothelial dysfunction in mice fed a high-cholesterol diet

Naoki Terasaka,1 Shuiqing Yu,2 Laurent Yvan-Charvet,1 Nan Wang,1 Nino Mzhavia,2 Read Langlois,1 Tamara Pagler,1 Rong Li,1 Carrie L. Welch,1 Ira J. Goldberg,2,3 and Alan R. Tall1

1Division of Molecular Medicine, 2Division of Cardiology, and 3Division of Preventive Medicine and Nutrition, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York, USA.

Plasma HDL levels are inversely related to the incidence of atherosclerotic disease. Some of the atheroprotective effects of HDL are likely mediated via preservation of EC function. Whether the beneficial effects of HDL on ECs depend on its involvement in cholesterol efflux via the ATP-binding cassette transporters ABCA1 and ABCG1, which promote efflux of cholesterol and oxysterols from macrophages, has not been investigated. To address this, we assessed endothelial function in Abca1+/−, Abcg1+/+, and Abca1−/−Abcg1−/− mice fed either a high-cholesterol diet (HCD) or a Western diet (WTD). Non-atherosclerotic arteries from WTD-fed Abcg1+/+ and Abca1+/−Abcg1+/− mice exhibited a marked decrease in endothelium-dependent vasorelaxation, while Abca1−/− mice had a milder defect. In addition, eNOS activity was reduced in aortic homogenates generated from Abcg1−/− mice fed either a HCD or a WTD, and this correlated with decreased levels of the active dimeric form of eNOS. More detailed analysis indicated that ABCG1 was expressed primarily in ECs, and that these cells accumulated the oxysterol 7-ketocholesterol (7-KC) when Abcg1+/− mice were fed a WTD. Consistent with these data, ABCG1 had a major role in promoting efflux of cholesterol and 7-KC in cultured human aortic ECs (HAECs). Furthermore, HDL treatment of HAECs prevented 7-KC–induced ROS production and active eNOS dimer disruption in an ABCG1-dependent manner. Our data suggest that ABCG1 and HDL maintain EC function in HCD-fed mice by promoting efflux of cholesterol and 7-oxysterols and preserving active eNOS dimer levels.

Introduction

Endothelial dysfunction is a key feature of early atherosclerotic lesions in both humans and animal models (1–3). It is characterized by decreased eNOS activity and NO bioavailability and increased expression of cell adhesion molecules such as VCAM-1 and ICAM-1, promoting atherosclerotic lesion formation, impaired blood flow, and thrombus formation. In animal models, increased dietary cholesterol plays a central role in inducing endothelial dysfunction (4–6). Dietary oxysterols, particularly 7-oxysterols, appear to have a key role in inducing decreased NO-induced vascular relaxation (7, 8). 7-Ketocholesterol (7-KC) is detected at high levels in human atherosclerotic plaques and in the plasma of patients with a high cardiovascular risk, and is abundant in oxidized LDL (9–11). In addition, oxysterols may be present in the diet and incorporated into plasma lipoproteins (12, 13). Dietary sources of oxysterols are cholesterol-rich foods (dairy, egg, meat products), especially those products that are heated in air during processing or are stored for long periods (14, 15). Thus, many foods in the Western diet (WTD) contain cholesterol oxidation products.

Plasma HDL levels are inversely related to the incidence of athero-thrombosis disease (16, 17). A part of the atheroprotective effect of HDL may be related to its role in preserving endothelial function (18, 19). The beneficial effects of HDL on ECs may include stimulation of proliferation, cell survival, migration, and NO synthesis as well as inhibition of the expression of VCAM-1 and ICAM-1 (20–23). HDL may have a specific role in reversing decreased eNOS activity in human ECs treated with oxidized LDL (24) or in reversing the decrease in eNOS-dependent vascular relaxation induced by high-cholesterol diets (HCDs) (4). The ability of HDL to cause relaxation of vascular rings has been reported to be impaired in scavenger receptor B-I–deficient (SR-BI–deficient) mice, and SR-BI expression in cultured cells enables an increase in eNOS activity in response to HDL through a mechanism that depends on the cholesterol efflux properties of HDL (25). While ATP-binding cassette transporters ABCA1 and ABCG1 have a major role in inducing cellular cholesterol efflux (26–28) and are known to be expressed in ECs (29), to our knowledge their role in preserving endothelial function has not been explored. ABCA1 mediates cholesterol efflux to lipid-poor apoA-I but only modestly increases cholesterol efflux to HDL (28, 30, 31). In contrast, ABCG1 promotes macrophage cholesterol efflux to HDL but not to lipid-poor apoA-I (28, 32–34). ABCG1 was recently shown to have a specific role not shared by ABCA1 in promoting efflux of 7-oxysterols from macrophages and transfected cells to HDL (28, 35). To better understand the adverse effects of dietary cholesterol and 7-oxysterols on endothelial function (7, 8), the present study was undertaken to test the hypothesis that ABCG1 and/or ABCA1, by promoting efflux of sterols and oxysterols from ECs, plays a key role in preserving eNOS activity in animals fed HCDs. Our studies show a major role for ABCG1 in defending endothelial eNOS activity in mice fed HCDs regulated to the efflux of cholesterol and 7-oxysterols and the preservation of eNOS dimer.

Nonstandard abbreviations used: CM-H2DCFDA, 6-carboxy-2,7-dichlorodihydrofluorescein diacetate, diacetoxymethyl ester; GSH, glutathione; HAEIC, human aortic EC; HCD, high-cholesterol diet; 7-KC, 7-ketocholesterol; i-NAMe, N-nitro-l-arginine methyl ester; NAC, N-acetylcysteine; SNP, sodium nitroprusside; SR-BI, scavenger receptor B-I; WTD, Western diet.

Conflict of interest: A.R. Tall has received consulting fees from Pfizer, Merck, AstraZeneca, and Roche; lecture fees from Merck; and grant support from Merck and Pfizer.

Citation for this article: J Clin Invest. 118:3701–3713 (2008). doi:10.1172/JCI35470.
Results
Impact of ABC transporter deficiency on endothelium-dependent vasorelaxation. Abcg1–/– and control mice were placed on a HCD (1.25% cholesterol, 7.5% cocoa butter, and 0.5% sodium cholate) for 11 weeks. After 11 weeks, both groups developed a similar moderate hypercholesterolemia (control, 331 ± 34 mg/dl; Abcg1–/–, 321 ± 46 mg/dl). To test vascular function in these mice, femoral arteries were preconstricted with phenylephrine (PE) and relaxant responses to endothelium-dependent ACh and endothelium-independent sodium nitroprusside (SNP) vasodilating agents were measured. Arterial vasorelaxation in response to ACh was markedly attenuated in Abcg1–/– mice (Figure 1A). The ACh dose-response curve was shifted to the right in Abcg1–/– mice, and the maximum relaxation response was significantly reduced compared with arteries from control mice (P < 0.01; Figure 1B). In contrast, there was no significant difference in relaxation in response to SNP (Figure 1C). There was also no significant difference in ACh-induced or SNP-induced arterial relaxation in WT and Abcg1–/– mice fed the Chow diet (Figure 1, D and E). We also assessed ACh-induced vascular relaxation in WTD-fed mice with single or combined deficiencies of Abca1 and Abcg1 (Figure 1, F and G). This revealed a similar severe defect in vascular relaxation in Abcg1–/– and Abca1–/–Abcg1–/– mice (EC50 79.6 ± 13.0 vs. 88.3 ± 14.7 nM), while the response of Abca1–/– mice (EC50 42.1 ± 11.8 nM) was intermediate between these groups and that of the controls (17.4 ± 2.7 nM) (Figure 1F). There was no difference in relaxation in response to SNP in any of the groups (Figure 1G). These findings suggest that while both transporters may be involved in preserving vascular relaxation responses, ABCG1 has a more prominent role than ABCA1.

Table 1 summarizes the effect of diet on vascular relaxation parameters in WT and Abcg1–/– mice. In the control group, the response to ACh was similar on the Chow or WTD (Table 1) but impaired in response to the HCD (Table 1). There was a progressively more marked impairment with increasing dietary cholesterol content in the Abcg1–/– mice (Table 1). The EC50 value for the response to ACh was approximately 4-fold greater than control in Abcg1–/– mice on the WTD, and 10-fold greater on the HCD, while there was no difference in response to the Chow diet (Table 1). The data indicate that ABCG1 plays a progressively more important role in maintaining endothelium-dependent vasorelaxation as dietary cholesterol content is increased.

Cholesterol and 7-KC accumulation in aorta: effects of diet and genotype. In view of the specific role of ABCG1 in efflux of 7-oxyesters from cells (35), we next measured the content of cholesterol and 7-KC in

Table 1

<table>
<thead>
<tr>
<th>Diet (cholesterol, %)</th>
<th>EC50 (nM) Control</th>
<th>Abcg1–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow (0.025)</td>
<td>27.7 ± 7.5</td>
<td>18.5 ± 3.4</td>
</tr>
<tr>
<td>WTD (0.25)</td>
<td>17.4 ± 2.7</td>
<td>79.6 ± 13.0</td>
</tr>
<tr>
<td>HCD (1.25)</td>
<td>72.4 ± 8.5a</td>
<td>696.2 ± 64.3b</td>
</tr>
</tbody>
</table>

The results are represented as mean ± SEM. *P < 0.05 vs. Chow diet. **P < 0.05 vs. control. n = 4–5 in each group.
non-atherosclerotic thoracic and abdominal aortas excluding the proximal aorta. Total cholesterol content was increased by the HCDs in a dietary cholesterol concentration–dependent manner (Figure 2A). However, there was no significant difference in cholesterol content between the control and Abcg1−/− mice (Figure 2A). 7-KC was not detectable in aortas of chow-fed mice but accumulated in response to the HCD (Figure 2B) and WTD (Figure 2, B and E). Accumulation of 7-KC was more prominent in Abcg1−/− mice (Figure 2, B and E). The ratio of 7-KC to cholesterol was significantly higher in Abcg1−/− mice than in controls in response to HCD (Figure 2C) and WTD feeding (Figure 2F). We also compared aortic cholesterol and 7-KC contents in Abcg1−/− mice with those in Abca1−/− and Abca1−/− Abcg1−/− mice in response to the WTD. There was no significant difference in cholesterol content between the control and Abcg1−/− or Abca1−/− mice (Figure 2D), while in Abca1−/− Abcg1−/− mice cholesterol content was significantly higher than in the controls (Figure 2D). In contrast, 7-KC was significantly increased in Abcg1−/− and Abca1−/− Abcg1−/− mice compared with controls, although no difference was found between Abcg1−/− and Abca1−/− Abcg1−/− (Figure 2E). The ratio of 7-KC to cholesterol was also significantly increased in Abcg1−/− and Abca1−/− Abcg1−/− mice (Figure 2F). Thus, we conclude that deficiency of both Abca1 and ABCG1 results in increased cholesterol accumulation compared with accumulation associated with a single deficiency of the transporters, while accumulation of 7-KC specifically reflects deficiency of ABCG1. The latter finding parallels the impairment of vasodilatory responses and suggests that the impaired Ach-induced vascular relaxation in Abcg1−/− mice could be brought about by aortic accumulation of 7-oxyesters.

eNOS protein expression and dimerization in aorta. Previous studies have shown that the formation of eNOS homodimers is necessary for eNOS activity (36, 37). In response to the HCD, eNOS dimer levels were dramatically reduced in Abcg1−/− mice (Figure 3, A and B). Total eNOS and phospho-eNOS levels were also moderately decreased in Abcg1−/− mice (Figure 3, C and D), but the ratio of phospho-eNOS to eNOS did not change. On the WTD, Abca1−/−, Abcg1−/−, and Abca1−/− Abcg1−/− mice exhibited decreased eNOS dimer levels in aortas (Figure 3, E and F). This reduction was most prominent in Abcg1−/− and Abca1−/− Abcg1−/− mice (Figure 3, E and F). There was no difference in eNOS or phospho-eNOS levels between the groups (Figure 3, G and H). In aortas of chow-fed mice, there was no significant difference in eNOS dimer levels, eNOS, or phospho-eNOS levels between the control and Abcg1−/− mice (Figure 3, I–L). PECAM levels were not changed in any groups or diets (Figure 3, C, G, and K), indicating an intact endothelium. These data suggest that endothelial dysfunction induced by ABCG1 deficiency in response to the HCD resulted from the reduction of eNOS dimer levels.

ABCG1 expression and accumulation of 7-KC in aorta. To further evaluate the role of ABCG1 in endothelium-dependent vasorelaxation, we investigated ABCG1 expression in non-atherosclerotic aorta of Abcg1−/− mice that harbor a lacZ cassette insertion at the Abcg1 locus. Blue nuclear lacZ expression was detected specifically in ECs (Figure 4A, arrowheads), but not in other cells indicated by nuclear fast red staining (Figure 4A, arrows). We also carried out PECAM staining in aorta of WTD-fed Abcg1−/− mice, which indicated an intact endothelium (Figure 4B). As expected, these segments of abdominal and thoracic aorta did not show any evidence of atherosclerosis or macrophage accumulation (data not shown). We also measured NOS activity using aortic lysates in WTD-fed WT and Abcg1−/− mice. The NOS activity in Abcg1−/− mice was significantly decreased (Figure 4C). These data are consistent with the reduction of eNOS dimer levels in aorta (Figure 3, E and F). We also isolated ECs from aorta in WTD-fed WT and Abcg1−/− mice using an affinity column with anti-PECAM antibody. After isolation of ECs, PECAM, eNOS, and Abcg1 mRNA levels were increased by 15- to 20-fold compared with the non-endothelial fraction (data not shown). There was no significant difference in cholesterol content between WT and Abcg1−/− mice (Figure 4D). 7-KC levels (Figure
4E) and the 7-KC/cholesterol ratio (Figure 4F) were significantly increased in the ECs isolated from Abcg1–/– mice, but not in the non-EC fraction. These findings suggest that lack of ABCG1 in ECs leads to 7-KC accumulation and reduced eNOS dimer levels and that decreased eNOS activity is responsible for impaired vascular relaxation in mice fed HCDs.

Effects of HDL and 7-KC on eNOS dimer and NOS activity. To further investigate the role of HDL and ABCG1 in promoting efflux of 7-oxysterols and preserving eNOS dimer levels and activity, we carried out experiments using human aortic ECs (HAECs), which are known to have a high level of ABCG1 (29). We first tested the effects of different concentrations of 7-KC (5–40 μg/ml) and HDL (100 μg/ml). 7-KC (5–40 μg/ml) significantly reduced eNOS dimer levels (Figure 5, A and B). Treatment of cells with HDL (100 μg/ml) following exposure to 7-KC prevented disruption of eNOS dimer levels by 7-KC (Figure 5, A and B). Only treatment with a high concentration of 7-KC (40 μg/ml) reduced eNOS and phospho-eNOS levels (Figure 5, A and C), and this did not change the ratio of phospho-eNOS to eNOS. The 7-KC concentration of 40 μg/ml also reduced eNOS mRNA (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI35470DS1) and induced apoptosis (Supplemental Figure 1B), but this was not observed at lower concentrations. Notably the concentration range of 5–10 μg/ml led to 7-KC levels that were comparable with those in isolated ECs from Abcg1–/– mice (see below). HDL treatment completely preserved eNOS dimer levels up to a concentration of 7-KC of 20 μg/ml (Figure 5, A and C). Increasing doses of 7-KC also progressively impaired eNOS activity, and this effect was reversed by HDL (Figure 5D). These data demonstrate a strong correlation between decreased eNOS dimer levels and NOS activity in response to increasing doses of 7-KC and show that both effects are reversed by HDL. 7-KC did not affect inflammatory gene expression such as Il6 or Mcp1 (Supplemental Figure 2). Insig1 and LDL receptor mRNA levels, which are regulated by SREBP-2, were reduced by 7-KC (Supplemental Figure 2). The reduction of these mRNAs most likely reflected intracellular accumulation of 7-KC.

Cholesterol and 7-KC mass efflux. Next, we measured cholesterol and 7-KC mass efflux to different acceptors in HAECs. HDL were loaded with cholesterol (5 μg/ml) and 7-KC (5 μg/ml) for 24 h. Before starting efflux, intracellular cholesterol and 7-KC contents were measured to determine the baseline contents for the control

Figure 3
Western blot for eNOS protein of mouse aorta. (A–D) Aortas from HCD-fed WT and Abcg1–/– mice. (E–H) Aortas from WTD-fed WT, Abca1–/–, Abcg1–/–, and Abca1–/–Abcg1–/– mice. (I–L) Aortas from chow-fed WT and Abcg1–/– mice. (A, E, and I) Western blot for eNOS dimer levels. (B, F, and J) Quantification of eNOS dimer/monomer levels. (C, G, and K) Western blot for eNOS and phospho-eNOS. (D, H, and L) Quantification of eNOS (filled bars) and phospho-eNOS (open bars). The results are represented as mean ± SEM. *P < 0.05 versus control.
Role of ABCG1 in the efflux of 7-oxysterols (35). To further evaluate the role of ABCG1 in the ability of HDL to promote 7-KC efflux and to protect ECs from mouse aortas. Original magnification, ×200 (A), ×100 (B). (C) Aortic NOS activity. (D) Cholesterol mass, (E) 7-KC mass, and (F) 7-KC/cholesterol ratio in ECs and non-ECs from mouse aortas. The results are represented as mean ± SEM.

Effects of ABCG1 and HDL on eNOS dimer levels. We examined the effects of different concentrations of HDL on eNOS dimer disruption by 7-KC. HDL treatment protected the disruption of eNOS dimer levels in a concentration-dependent manner with concentrations between 25 and 100 μg/ml (Figure 7, A and B). The reduction of eNOS by 7-KC required a relatively long incubation time (>4 h) (Figure 7, C and D). We have previously reported a specific role of ABCG1 in the efflux of 7-oxysterols (35). To further evaluate the role of ABCG1 on eNOS dimer levels, we tested the effects of different oxysterols and cholesterol (each 10 μg/ml) in similar experiments. 7β-Hydroxycholesterol as well as 7-KC significantly decreased eNOS dimer levels (Figure 7, E and F). This pattern of predominant effects of 7-oxysterols on eNOS dimer levels parallels the specific role of ABCG1 in promoting efflux of these oxysterols compared with other sterols (35).

To assess the specific role of ABCG1 in 7-KC–induced disruption of eNOS dimer levels, we knocked down expression of ABCG1 by siRNA. In ABCG1 siRNA-transfected HAECS, the protective effect of HDL was abolished (Figure 7, G and H). In contrast, suppression of neither ABCA1 nor SR-BI affected the ability of HDL to protect against disruption of eNOS dimer levels by 7-KC (Figure 7, G and H). These experiments show a specific requirement for ABCG1 in the ability of HDL to promote 7-KC efflux and to protect ECs from eNOS dimer disruption induced by 7-KC.

Effects of ABCG1 and HDL on ROS production. Previous studies have shown that disruption of eNOS dimer levels can be mediated by peroxynitrite (ONOO−), which is generated from superoxide (O2−) and NO (38). To investigate the hypothesis that HDL and ABCG1 reverse the effects of 7-KC on ROS production, we used the cell-permeable reagent 6-carboxy-2,7-dichlorodihydrofluorescein diacetate, diacetoxymethyl-ester (CM-H2DCFDA) (39). In HAECS, 7-KC (5–40 μg/ml) induced ROS formation in a dose-dependent manner (Figure 8, A and B). The ROS production by 7-KC required more than 4 h incubation (Figure 8C). The concentration dependence and the time-course response paralleled those of eNOS dimer disruption induced by 7-KC (Figure 7, A–D). 7β-Hydroxycholesterol (10 μg/ml) also significantly increased ROS (P < 0.01), whereas cholesterol, 7α-hydroxycholesterol, 25-hydroxycholesterol, or 27-hydroxycholesterol did not alter eNOS dimer levels (Figure 7, E and F).
Similar protective effects of HDL and ABCG1 on ROS production by 7-KC were observed in primary mouse aortic ECs isolated in a manner similar to the studies described in Figure 4, D–F (see also Supplemental Figure 3). We also measured NOS activity in the similar experiments. HDL preserved the reduction of NOS activity by 7-KC, whereas the HDL protection was abolished by ABCG1 siRNA transfection (Figure 8F). These experiments suggest HDL protects against 7-KC-induced ROS production (data not shown).

To further analyze the mechanism of eNOS dimer disruption by 7-KC, we investigated the effect of NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). L-NAME treatment prevented eNOS dimer disruption by 7-KC in a dose-dependent manner (Figure 9, E and F). We also investigated the effect of 7-KC on protein tyrosine nitrosylation, since nitrotyrosine formation is considered an indicator for ONOO− production. Treatment with 7-KC significantly increased the detection of nitrotyrosine-positive protein (Figure 9G). In addition, either the presence of L-NAME or HDL significantly reduced the level of nitrotyrosine formation (Figure 9G). These data strongly suggest that 7-KC induces formation of O2·−, which reacts with eNOS-generated NO to form ONOO−, which in turn leads to eNOS oxidation.

**Effect of apoA-I transgene expression in endothelial function.** To further evaluate the role of HDL in endothelial function, we also investigated the effect of apoA-I transgene expression on endothelium-dependent vasorelaxation in HCD-fed Ldlr−/− mice. ApoA-I transgene expression significantly improved endothelium-dependent vasorelaxation (EC50 Ldlr−/−, 84.4 ± 11.6 vs. Ldlr−/−apoA-I Tg, 23.4 ± 6.5 nM; P < 0.05) (Figure 10A). There was no difference between the groups in the response to SNP (Figure 10B). ApoA-I transgenic expression also significantly increased eNOS dimer levels (Figure 10, C and D) and NOS activity (Figure 10F). There was no difference between the groups in eNOS and phospho-eNOS levels (Figure 10, C and E). We also measured cholesterol and 7-KC contents in the aorta. In Ldlr−/−apoA-I Tg mice, both cholesterol (Figure 10G) and 7-KC (Figure 10H) contents were significantly decreased, but the magnitude of 7-KC reduction was more pronounced. These data suggest that increased HDL levels resulting from apoA-I transgene expression promote efflux of 7-KC from the aorta, contributing to preservation of eNOS dimer levels and activity.

**Discussion**

One of the most important athero-protective functions of HDL is thought to be the stimulation of macrophage cholesterol efflux, and recent studies have highlighted the key roles of ABCA1 and ABCG1 in reversing macrophage foam cell formation (40) and atherosclerosis (41, 42). HDL has also been shown to exert 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 and reverses endothelial dysfunction (20–22). Our studies revealed a non-redundant role of ABCG1 and a lesser role of ABCA1 in preserving endothelial eNOS activity in mice fed HCDs, and our results suggest that this may be a major mechanism underlying the ability of HDL to defend endothelial NO activity in response to such diets. The ability of
ABCG1 to preserve endothelial function appears to be at least partly related to its role in promoting efflux of 7-oxysterols such as 7-KC to HDL.

HDL has consistently been shown to increase eNOS-dependent NO activity in cultured ECs (24, 25) and aortic rings (43) and in human forearm blood flow studies (44, 45). In humans, HDL levels are correlated with flow-mediated vasodilation responses of the brachial artery (18, 19) and with decreased coronary vasoconstrictor responses (44). Importantly, infusion of recombinant phospholipid/apoA-I particles into Tangier disease heterozygotes with isolated low HDL levels reversed defective forearm blood flow measurements (46). Deckert et al. (7, 8) showed that 7-oxysterols can produce decreased eNOS activity in rabbits and HUVECs. In apoE/− mice fed a chow diet, arterial eNOS activity was preserved but became impaired when mice were challenged with a HCD (4). Importantly, apoA-I transgene expression reversed the decrease in eNOS activity induced by the HCD (4). The current study in HCD-fed Ldlr−/− mice reproduced preservation of endothelium-dependent aortic relaxation by apoA-I transgene overexpression (Figure 10), and we show that this effect is associated with preservation of eNOS dimer levels and eNOS activity and reduced aortic 7-KC levels.

Our studies extend these important earlier observations (4, 7, 8) and suggest that the underlying mechanism by which increased or basal HDL levels protect the endothelium involves efflux of dietary sterols, especially 7-oxysterols from ECs to HDL, mediated principally by ABCG1 (Figure 11). It is most likely that dietary oxysterols are normally incorporated into chylomicrons, cleared by the liver, converted into bile acids, and excreted (13). However, when there is delayed clearance of chylomicron remnants, as occurs in apoE/− mice or in humans with increased coronary heart disease risk (47), the vascular endothelium has increased exposure to dietary oxysterols, and ABCG1 and HDL likely have a key role in excluding or promoting efflux of 7-oxysterols from ECs. Indeed, we found that ABCG1 was expressed specifically in endothelium in non-atherosclerotic mouse aorta (Figure 4A) and 7-KC also accumulated in ECs isolated from the aorta in Abcg1−/− mice (Figure 4E). Even though HDL may have a variety of different antioxidant properties in different settings (48, 49), the ability of HDL to promote efflux of 7-KC, reduce ROS production, and preserve eNOS dimer levels and activity were all dependent on ABCG1 expression, indicating that the underlying mechanism involves ABCG1-mediated oxysterol efflux. Notably, while 7-KC was readily detected in non-lesioned arteries from mice fed HCDs, it was not measurable in arteries from mice fed chow diets (Figure 2), making it unlikely that 7-KC was artifically formed during sample processing. Moreover, 7-KC was specifically increased as a result of ABCG1 deficiency (Figure 2), consistent with the role of ABCG1 and not ABCA1 in promoting efflux of this oxysterol to HDL (35).

In the present study in HAECS, disruption of eNOS dimer levels was induced by a 7-KC concentration of 5 μg/ml, which might be equivalent to levels found in human plasma after a fat-rich meal (50). Intracellular 7-KC content in HAECS treated with the relevant concentrations of 7-KC (5–10 μg/ml) were around 10 μg/mg protein, approximating the concentration found in isolated ECs from aortas in WTD-fed Abcg1−/− mice (Figure 4F), in which eNOS dimer levels were reduced (Figure 3). Thus, these 7-KC concentrations are likely sufficient to induce endothelial dysfunction. The current findings agree with the notion that endothelial dysfunction is a key feature of early atherosclerosis (1) and also occurs transiently in the postprandial state (51).

Our parallel studies in mice and in HAECS suggest that ABCG1 mediates the efflux of 7-oxysterols from ECs to HDL, resulting in decreased ROS formation and preservation of the active dimeric form of eNOS (Figure 9). O2− is known to inactivate NO and generate ONOO− (37, 38). ONOO− can disrupt eNOS dimers through oxidation and displacement of the zinc metal ion (37, 52). Our studies also demonstrate that both t-NAME and antioxidants reversed the disruption of eNOS dimer levels by 7-KC (Figure 9). These data strongly suggest that 7-KC induced O2− and ONOO− production through interaction with NO, resulting in eNOS oxidation (Figure 11). There is considerable evidence that increased ROS can inhibit eNOS dimer formation and produce endothelial dysfunction in vivo, for example in diet-induced diabetic mice (53, 54) or in apoptosis signal–regulating kinase-1–deficient mice (55).

A number of different mechanisms have been proposed to account for the ability of HDL to preserve or increase arterial eNOS activity. HDL appears to be moderately effective in inducing eNOS-dependent vascular relaxation when directly added to or.
tic rings isolated from rats or mice (43, 56). However, the effect is very rapid (within a few minutes) and is saturated at very low concentrations of HDL (10 μg/ml), far below that normally bathing the endothelium (43). The response to added HDL is defective in vascular rings isolated from chow diet–fed SR-BI–/– mice (43), and from mice lacking the lysophospholipid S1P3 receptor (56). The direct effect of HDL on induction of eNOS activity has also been attributed to minor components such as lysophospholipids (56) or estrogen (57), but the concentrations of these components may not be sufficiently high to be physiologically relevant (25). While SR-BI may not have a major role in mediating net cellular cholesterol efflux to HDL in vivo, it is likely that ABCA1 and ABCG1 do mediate net efflux (27, 42, 58). Finally, our study has not assessed the role of ABC transporters in efflux to HDL of oxidized phospholipids, which are also likely to be important in endothelial dysfunction (59–61). Further studies are required to assess the relative roles of these different potential mechanisms in HDL-induced eNOS activity in vivo. Our study conclusively demonstrates the essential role of the ABC transporters, and especially ABCG1, in this process and delineates one mechanism involving efflux of 7-oxysterols and preservation of eNOS dimer levels.

Therapies that increase HDL levels, such as niacin and cholesterol ester transfer protein inhibitors, probably activate the ABCG1-cholesterol/oxysterol efflux pathway not only in macrophages (26–28, 35) but also in ECs, likely with beneficial effects on endothelial function. Importantly, niacin therapy has been shown to improve
NO-mediated vascular relaxation in humans (62). Our studies suggest that the underlying mechanism may involve increased efflux of cholesterol and 7-oxyesters via the ABCA1 and ABCG1 pathway.

**Methods**

**Materials.** The ROS-sensitive fluorescent probe CM-H<sub>2</sub>DCFDA and nuclear fast red were from Invitrogen. Anti-eNOS and anti–phospho-eNOS (S1177) antibodies were obtained from BD Transduction Laboratories. Anti-ABCG1, anti–PECAM, and anti-nitrotyrosine antibodies were purchased from Abcam. SR-BI antibody was from Santa Cruz Biotechnology Inc. Anti-β-actin antibody, X-gal (5-bromo-4-chloro-3-indolyl β-d-galactopyranoside), lipoprotein-deficient serum, NAC, GSH, phenylephrine, ACh, SNP, cholesterol, 7-KC, 7β-hydroxycholesterol, and 25-hydroxycholesterol were purchased from Sigma-Aldrich. 27-Hydroxycholesterol was obtained from Steraloids. l-NAME was purchased from Cayman Chemical. Human apoA-I was obtained from Biodesign International. HDL (density 1.063–1.210 g/ml), HDL<sub>2</sub> (density 1.063–1.125 g/ml), and HDL<sub>3</sub> (density 1.125–1.210 g/ml) were isolated by preparative ultracentrifugation from normolipidemic human plasma and stored in PBS.

**Mouse studies.** Abcg1<sup>−/−</sup>, Abca1<sup>−/−</sup>, and Abca1<sup>−/−</sup>Abcg1<sup>−/−</sup> mice have been previously described (41). We performed studies with a chow diet (0.025% cholesterol), a HCD (1.25% cholesterol, 7.5% cocoa butter, and 0.5% sodium cholate; catalog no. TD88051; Harlan Teklad) and a WTD (21% milk fat, 0.2% cholesterol; catalog no. TD88137; Harlan Teklad).

**Figure 8**

Effects of ABCG1 and HDL in ROS production by 7-KC. (A and B) HAECs were incubated with 7-KC (1–40 μg/ml) for 16 h. Intracellular ROS was determined after 30 min of pulse, using CM-H<sub>2</sub>DCFDA. (A) Fluorescence of CM-H<sub>2</sub>DCFDA in HAECs. (B) Quantification of CM-H<sub>2</sub>DCFDA fluorescence. (C) Fold increase in fluorescence over time with 7-KC treatment. *P < 0.05 compared with no 7-KC at same time point. (D–F) HAECs were transfected with scrambled or ABCG1 siRNA. Forty-eight hours after transfection, HAECs were treated with 7-KC (10 μg/ml) in the presence or absence of HDL (100 μg/ml) for 16 h. (D) Fluorescence of CM-H<sub>2</sub>DCFDA. (E) Quantification of CM-H<sub>2</sub>DCFDA fluorescence. Inset: Western blot for ABCG1. (F) NOS activity. The results are represented as mean ± SEM of 3 individual experiments. Original magnification, ×200. *P < 0.05 versus control.
C57BL/6 Ldlr−/− mice and C57BL/6 apoA-I Tg mice (63) were obtained from the Jackson Laboratory and crossed to generate Ldlr+/− apoA-I Tg mice. Next, these animals were crossed with DBA/1LacJ mice (The Jackson Laboratory) to obtain the genetically uniform F1 generation. F1 hybrid C57BL/6 × DBA Ldlr+/− apoA-I Tg mice were put on the HCD.

Animals had ad libitum access to both food and water. Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Tissue collection. Mice were anesthetized with an intraperitoneal injection of ketamine. The chest and peritoneal cavity were opened and the circulatory system was perfused via the left ventricle with PBS. Aortas were removed and processed for all assays. For vascular studies, the left superficial femoral artery was removed and immediately placed in ice-cold physiologic salt solution.

Vascular function studies. Femoral arteries with intact endothelium and similar dimensions were mounted on a small vessel wire myograph (Danish MyoTechnology) as described previously (53). Vessels were bathed in physiologic salt solution at 37°C and aerated continuously with 5% CO2/95% O2 to achieve pH 7.4. The startup protocol and evaluation of vessel viability was conducted as described previously (53). Concentration response curves were performed for ACh (endothelium dependent) and SNP (endothelium-independent NO-releasing agent). Wall tension was expressed as mN/mm of artery length. Sensitivity to the agonist was expressed as the negative log of EC50 (−log EC50). Sensitivity was calculated from each concentration response curve by fitting the Hill equation using Prism (GraphPad Software).

Isolation of ECs from aorta. Mice aortas were perfused with PBS and digested in RPMI 1640 medium containing collagenase D (2 mg/ml; Roche Applied Science) at 37°C for 45 min. The digest was sequentially filtered through 100-μm, 70-μm, and 40-μm cell strainers and was washed with PBS. The cells were incubated with anti-PECAM biotin-conjugated

Figure 9
Effects of antioxidants and NOS inhibitor on eNOS dimer disruption by 7-KC. (A–D) HAECs were incubated with 7-KC (10 μg/ml) in the presence of GSH (10 mM), NAC (10 mM), or HDL (100 μg/ml) for 16 h. (A) Western blot for eNOS dimer and monomer. (B) Quantification of the eNOS dimer/monomer ratio. (C) Fluorescence of CM-H2DCFDA. Original magnification, ×200. (D) Quantification of CM-H2DCFDA fluorescence. (E and F) HAECs were incubated with 7-KC in the presence or absence of L-NAME for 16 h. (E) Western blot for eNOS dimer and monomer. (F) Quantification of the eNOS dimer/monomer ratio. (G) HAECs were incubated with 7-KC (5–20 μg/ml) in the presence of L-NAME or HDL (100 μg/ml) for 16 h. Western blot for nitrotyrosine. All lanes were run on the same gel but were noncontiguous between 7-KC and 7-KC + L-NAME. The results are represented as mean ± SEM of 3 individual experiments. *P < 0.05 versus vehicle control; †P < 0.05 versus 7-KC alone. Veh, vehicle.
antibody (Millipore) at 4°C for 15 min and were washed with PBS. Next, the cells were labeled with streptavidin microbeads (Miltenyi Biotec) and aortic ECs were separated by MACS column (Miltenyi Biotec) according to the manufacturer’s instructions. Isolated aortic ECs were used for sterol mass measurement.

LacZ expression and PECAM immunostaining. The tissues were snap-frozen in OCT and stored at –80°C. Frozen sections 10 μm long were prepared. To determine β-galactosidase activity, the glass slides were incubated for 16 h in the presence of X-gal. The slides were counterstained with nuclear fast red. PECAM immunostaining was carried out as previously described (64).

Cell culture. HAECs and the culture medium EMG-2 were purchased from Lonza. The cells were grown in EMG-2 at 37°C in humidified 5% CO₂ and used for experiments between passages 3 and 5. All siRNAs were purchased from Invitrogen or Santa Cruz Biotechnology Inc. HAECs were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. Forty-eight hours after transfection, HAECs were treated with 7-KC in the presence or absence of HDL.

Sterol mass analysis. The lipid fractions of abdominal aortas, isolated ECs or non-ECs from aorta, and HAECs were extracted using hexane/isopropanol (3:2 vol/vol) in presence of stigmasterol added as the internal standard. Total cholesterol and 7-KC were determined after saponification by gas-liquid chromatography (26, 35).

Sterol mass efflux assay. HAECs were incubated in EGM-2 plus 5% lipoprotein-deficient serum with cholesterol (5 μg/ml) and 7-KC (5 μg/ml) for 24 h. The next day, cells were washed with PBS and then incubated in EGM-2 plus 5% lipoprotein-deficient serum alone or supplemented with human apoA-I or HDL for 16 h. After the efflux period, media and cells were collected separately and lipids were extracted with hexane/isopropanol (3:2 vol/vol) with stigmastanol as the internal standard. Sterol mass of media and cells was determined using gas chromatography. Percentages of sterol mass efflux were calculated by the ratio of sterol mass in the medium to total (medium plus cellular) sterol mass.

NOS activity assay. The NO synthesizing activity was determined by quantifying the rate of the conversion of [³H]-arginine to [³H]-citrulline with kits obtained from Calbiochem-Novabiochem according to the manufacturer’s instructions (52).

Western blotting. Protein was resolved on 4%–20% SDS-PAGE reducing gels (Bio-Rad). Protein was transferred to PVDF membranes and probed with antibody (Millipore) at 4°C for 15 min and were washed with PBS. Next, the cells were labeled with streptavidin microbeads (Miltenyi Biotec) and aortic ECs were separated by MACS column (Miltenyi Biotec) according to the manufacturer’s instructions. Isolated aortic ECs were used for sterol mass measurement.

LacZ expression and PECAM immunostaining. The tissues were snap-frozen in OCT and stored at –80°C. Frozen sections 10 μm long were prepared. To determine β-galactosidase activity, the glass slides were incubated for 16 h in the presence of X-gal. The slides were counterstained with nuclear fast red. PECAM immunostaining was carried out as previously described (64).

Cell culture. HAECs and the culture medium EMG-2 were purchased from Lonza. The cells were grown in EMG-2 at 37°C in humidified 5% CO₂ and used for experiments between passages 3 and 5. All siRNAs were purchased from Invitrogen or Santa Cruz Biotechnology Inc. HAECs were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. Forty-eight hours after transfection, HAECs were treated with 7-KC in the presence or absence of HDL.

Sterol mass analysis. The lipid fractions of abdominal aortas, isolated ECs or non-ECs from aorta, and HAECs were extracted using hexane/isopropanol (3:2 vol/vol) in presence of stigmasterol added as the internal standard. Total cholesterol and 7-KC were determined after saponification by gas-liquid chromatography (26, 35).
Circulation.


6. Shudo, K., et al. 1990. Test. Bonferroni post-hoc tests were utilized. Results are represented as means ± SEM.


45. Bansal, S., et al. 2007. Fasting compared with non-