Adenosine Monophosphate Activated Protein Kinase Regulates ABCG1-Mediated Oxysterol Efflux From Endothelial Cells and Protects Against Hypercholesterolemia-Induced Endothelial Dysfunction

Dan Li, Yuan Zhang, Jing Ma, Wenhua Ling, Min Xia

Objective—Adenosine monophosphate activated protein kinase (AMPK) has been identified as a regulator of vascular function via the preservation of endothelial cell (EC) function. In this study, we examined whether the beneficial effects of AMPK on ECs are dependent on its involvement in cholesterol efflux and its impact on hypercholesterolemia-induced endothelial dysfunction.

Methods and Results—Using human aortic ECs and bovine aortic ECs, we show that AMPK activation upregulates ATP binding cassette G1 (ABCG1) expression independently of liver X receptor alpha (LXRs) transactivation activity but through a posttranscriptional mechanism that increases mRNA stability. Using a heterologous system and a luciferase reporter, we further identify that the 3' untranslated region of the ABCG1 mRNA is responsible for the regulatory effects of AMPK activation. 5-Aminoimidazole-4-carboxamide-1-beta-d-ribose treatment promotes endothelial 7-ketocholesterol efflux and prevents 7-ketocholesterol (7-KC)-induced reactive oxygen species production in an ABCG1-dependent manner, thus preserving endothelial nitric oxide synthase activity and nitric oxide bioavailability. Notably, in vivo studies using C57BL/6J mice receiving a high-cholesterol diet revealed that the infusion of 5-aminoimidazole-4-carboxamide-1-beta-d-ribose increases vascular ABCG1 expression and improves vascular reactivity. These effects are abrogated by the AMPK antagonist compound C by the vascular gene transfer of ABCG1 small interfering RNA.

Conclusion—Our current findings uncover a novel mechanism by which AMPK protects against hypercholesterolemia-mediated endothelial dysfunction. (Arterioscler Thromb Vasc Biol. 2010;30:1354-1362.)

Key Words: ABC transporter ▪ endothelial function ▪ vascular biology ▪ AMP-activated protein kinase

Endothelial dysfunction, which manifests as impaired endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) bioavailability, is a key feature of early atherosclerosis.1-4 A growing body of evidence has now implicated increased dietary oxysterols as a potent inhibitor of endothelium-dependent vascular relaxation and a significant contributor to the initiation and development of endothelial dysfunction, which suggests a vital link between hypercholesterolemia and the formation of atherosclerotic lesions.5-7 Oxysterols are oxygenated forms of cholesterol that are present at low levels in the circulatory system and accumulate in plasma and tissues in some diseases. In characteristic lesions of atherosclerosis, 7-oxygenated oxysterols, predominantly 7-ketocholesterol (7-KC), are detectable at high levels and have been implicated in the pathology of this disease.8,9

Adenosine monophosphate activated protein kinase (AMPK) is a member of a metabolite-sensing protein kinase family, contains a catalytic alpha subunit and regulatory beta and gamma subunits, and functions as a protein serine/threonine kinase.10 The activation of AMPK attenuates anabolic processes, such as the synthesis of proteins, fatty acids, and cholesterol, but stimulates ATP-generating catabolic pathways.11 Recent findings further suggest that AMPK is also present in the endothelium and maintains endothelial homeostasis by regulating eNOS activity and increasing NO bioactivity.12,13 Hence, the bulk of the available data link AMPK signaling and endothelial function. However, the impact of AMPK activation on hypercholesterolemia-induced endothelial dysfunction and the involved underlying molecular mechanism remains unknown.
The ATP-binding cassette transporters ABCA1 and ABCG1 play an important role in inducing cellular cholesterol efflux\(^\text{14-16}\) and are known to be expressed in vascular endothelial cells (ECs).\(^\text{17}\) More importantly, ABCG1 was recently shown to have a specific role not shared by ABCA1 in promoting the efflux of 7-oxysterols from ECs\(^\text{18}\) and to markedly reverse hypercholesterolemia-induced endothelial dysfunction.\(^\text{19}\) Because of the essential role of ABCG1 in regulating cholesterol homeostasis in vascular ECs, our present study was undertaken to test the hypothesis that AMPK, by promoting the ABCG1-mediated efflux of sterols and oxysterols from ECs, plays a key role in preserving eNOS activity and thus improving endothelial dysfunction caused by AMPK.

**Materials and Methods**

**Cell Culture**

Human aortic ECs (HAECs) were cultured in M199 medium supplemented with FBS (20%), penicillin (100 U/mL), streptomycin (100 μg/mL), heparin (90 μg/mL), and EC growth supplement (20 μg/mL). The cells were grown at 37°C in humidified 5% CO\(_2\) and used for experiments between passages 3 and 5. For detailed methods, see the supplement, available online at http://atvb.ahajournals.org.

**Results**

**AMPK Activation Upregulates ABCG1 Expression in Vascular ECs**

To evaluate the effects of AMPK activation on ABCG1 expression, we exposed HAECs to the AMPK activator 5-aminoimidazole-4-carboxamide-1-D-ribose (AICAR) (0 to 2 mmol/L) for 2 to 24 hours and examined the effects on ABCG1 expression. AICAR treatment was found to cause a time-dependent increase in the expression of ABCG1 mRNA as determined by Northern blot (Figure 1A) and real-time RT-PCR (Figure 1B). The figure shown is representative of 3 independent experiments. The abundance of ABCG1 mRNA in untreated control cells was defined as 1, and the amounts of ABCG1 mRNA from AICAR-treated cells were then expressed as fold changes in this control value. *P<0.05, **P<0.01 versus 0 hours. C and D, Dose-dependent induction of ABCG1 mRNA expression. HAECs were treated with AICAR for 8 hours at the indicated concentrations, and total RNA was isolated for the analysis of ABCG1 and GAPDH mRNA expression by Northern blot (C) and real-time polymerase chain reaction (D). *P<0.05, **P<0.01 versus 0 mmol/L. E, ABCG1 expression was measured by Western blot. F, The uptake of dioctadecylindocarbocyaniniodide (DiI)-HDL was measured by FACScan with 1×10^5 cells per sample. The mean fluorescence intensity (MFI) of untreated cells is expressed as 100%. The data shown are expressed as a percentage of the control from 3 independent measurements.
0.25 mmol/L AICAR, and a maximal 4-fold increase over the control was seen with a concentration of 2 mmol/L. Increases of similar magnitudes in the ABCG1 mRNA levels could be confirmed by quantitative RT-PCR (Figure 1D). Western blot analysis also demonstrated that AICAR induces ABCG1 protein expression in a concentration-dependent fashion (Figure 1E).

Unrelated AMPK activators, such as metformin20 and A-769662,21 were also shown to increase ABCG1 mRNA and protein expression in HAECs (Supplemental Figure IA and IB). Furthermore, the overexpression of a constitutively active form of AMPKα (Ca-AMPKα) led to a robust increase in ABCG1 expression that could not be further enhanced by AICAR. In contrast, when a dominant-negative form of AMPKα (Dn-AMPKα) was overexpressed, AICAR was unable to enhance ABCG1 expression (Supplemental Figure IC). The effects of AICAR on ABCG1 were further confirmed in bovine aortic ECs in which a similar increase in ABCG1 mRNA levels was observed (Supplemental Figure ID and IE). The enhancement of ABCG1 expression by AICAR was further found to directly translate into increased functional capacity, as it induced the binding of 3,3'-dioctadecylindocarbocyaniniodide–high-density lipoprotein (HDL) to HAECs in a dose-dependent manner (Figure 1F). This was almost completely abrogated following AMPK inhibition by compound C or Dn-AMPKα (Supplemental Figure IF). The activation of AMPK thus triggers ABCG1 expression.

AMPK Activation Regulates ABCG1 mRNA Stability via Its 3'-Untranslated Region

To elucidate the mechanism by which AMPK activation increases ABCG1 expression, we analyzed the impact of the AMPK agonist AICAR on LXRE response element-dependent transcriptional activity by transfecting HAECs with an liver X receptor element (LXRE)-driven luciferase-reporter vector (LXRE-tk-Luc). Following transfection, cells were treated with AICAR (1 mmol/L) for 24 hours, and actinomycin D (Act D; 5 μg/mL) was added to the cells for different intervals. Total RNA was then isolated and ABCG1 mRNA was assayed by Northern blot. The normalized ABCG1 mRNA signals were plotted as the percentage of the ABCG1 mRNA remaining. Decay curves were plotted versus time. E, Ten hours after transfection with a pGL3 control vector and pGL3-containing human ABCG1 3'-UTR, HAECs were treated with AICAR (1 mmol/L) or vehicle (control) for 24 hours. The cells were then lysed, and a luciferase assay was performed. The results from 3 independent experiments are presented as the mean±SEM.
These results prompted us to then investigate the possibility that AICAR treatment may stabilize ABCG1 mRNA, resulting in higher expression levels. Actinomycin D was added to both vehicle- and AICAR-treated HAECs for specified periods, and the half-life of ABCG1 mRNA was determined by Northern blot in each case (Figure 2C). Based on the observed decay values, we found that AICAR prolongs the turnover rate of ABCG1 transcripts by approximately 3-fold (182 minutes versus 60 minutes; Figure 2D). In contrast, the mRNA stability of GAPDH was unaltered by AICAR.

To further explore these stabilization mechanisms, we investigated whether AMPK modulates 3′-untranslated regions (3′-UTRs), which have been shown to destabilize mRNA. As shown in Figure 2E, the presence of the ABCG1 3′-UTR attenuates the baseline luciferase activity compared with an empty vector, and AICAR treatment restores this reporter activity. This indicates that the 3′-UTR of human ABCG1 plays a role in the mRNA stabilization induced by AMPK.

**AMPK Activation Promotes Cholesterol and 7-KC Mass Efflux**

We next investigated the role of AMPK activation in ABCG1-mediated cholesterol and 7-KC efflux. AICAR treatment induced both cholesterol and 7-KC mass efflux in a dose-dependent fashion in the presence of acceptor HDL (Figure 3A) which was significantly abolished by compound C or following Dn-AMPK transfection (Figure 3B). This indicates an AMPK dependence of AICAR-stimulated cholesterol efflux. We next examined the effects of the knockdown of ABCG1 and ABCA1 by small interfering RNA (siRNA) transfection on AICAR-mediated cholesterol and 7-KC mass efflux. Western blot analysis revealed that ABCG1 and ABCA1 expression is effectively suppressed by siRNA (Supplemental Figure II). The suppression of ABCG1 expression by the specific RNA interference (RNAi) significantly reduced both cholesterol and 7-KC mass efflux by AICAR, but ABCA1 silencing had no such effect (Figure 3C). These results indicate that ABCG1 is a critical regulator of the cholesterol and oxysterol efflux induced by AMPK activation.

**AMPK Activation Modulates Oxidative Stress and Preserves eNOS Activity**

To determine the consequences of AICAR-induced oxysterol efflux on oxidant stress, we measured 6-carboxy-2′,7′-dichlorodihydrofluorescein fluorescence, as this compound is a nonspecific marker of reactive oxygen species (ROS) accumulation.22 Compared with vehicle-treated cells, these cells were then washed with PBS and incubated with HDL (20 μg/mL) as an acceptor for a further 16 hours. The results are represented as the mean±SEM of 3 individual experiments. *P<0.05, **P<0.01 versus control (cholesterol [Chol]); #P<0.05, ##P<0.01 versus control (7-KC). B, 7-KC–loaded HAECs were pretreated with the AMPK inhibitor compound C (10 mmol/L) for 2 hours or transfected with Dn-AMPK. The cells were then incubated with AICAR (2 mmol/L) for a further 24 hours. Results are represented as mean±SEM of 3 individual experiments. *P<0.01 versus AICAR (Chol); #P<0.01 versus AICAR (7-KC). C, HAECs were transfected with ABCG1 siRNA, ABCA1 siRNA, or a nonspecific control siRNA. Twenty-four hours after transfection, the cells were further incubated with 7-KC (10 μg/mL) alone or in the presence of AICAR (1 mmol/L) for 24 hours. HAECs were then washed with PBS and incubated with or without HDL (20 μg/mL) for 16 hours. Results are represented as mean±SEM of 3 individual experiments. *P<0.05 (Chol); #P<0.05 (7-KC) between AICAR+control siRNA and AICAR+ABCG1 siRNA.
posed to 7-KC demonstrated increased ROS levels (Figure 4A), which were found to be associated with a concomitant decrease in the reduced glutathione to oxidized glutathione ratio (Figure 4B). Exposure to 7-KC also caused a significant decrease in eNOS activity (Figure 4C), whereas AICAR treatment reversed 7-KC mediated oxidative stress and eNOS disruption. We next examined the cGMP levels as a measure of the bioavailability of NO23,24 and found that they were markedly decreased in 7-KC-treated HAECs compared with the control cells. However, when stimulated with AICAR, 7-KC-treated cells demonstrated a significant increase in cGMP production (Figure 4D). ABCG1 suppression abrogated the ability of AICAR to protect against eNOS inhibition (Figure 4C) and reduction of NO bioavailability by 7-KC (Figure 4D), indicating the essential role of ABCG1 in the AICAR-mediated inhibition of oxidative stress and protection of eNOS activity.

**AICAR Treatment Increases ABCG1 Expression, Reduces Cholesterol/7-KC Accumulation in the Aorta and Improves Endothelium-Dependent Vasorelaxation**

To determine whether our in vitro findings could be reproduced in vivo, we treated normal cholesterol diet (NCD)- and HCD-fed C57BL/6J mice with AICAR or vehicle for 12 weeks. This treatment significantly increased the levels of phosphorylated AMPK Thr172 in the aorta compared with the control mice (Supplemental Figure IIIA). AMPK activation by AICAR was also confirmed by AMPK activity analysis using the SAMS peptide as a substrate25 (Supplemental Figure IIIB). Simultaneously, AICAR treatment was found to markedly enhance ABCG1 protein expression in the aorta compared with untreated mice (Supplemental Figure IVA and IVB), and this was accompanied by a marked decrease in the total cholesterol mass (Supplemental Figure IVC) and 7-KC mass (Supplemental Figure IVD) in AICAR-treated mice. We further evaluated the vascular reactivity to examine the functional consequences of AMPK activation. Endothelium-dependent relaxation following exposure to acetylcholine (ACh) was markedly impaired in mice on HCD compared with mice on NCD. AICAR treatment also caused a striking improvement in aortic endothelium-dependent vasorelaxation in HCD-fed mice, indicative of a significant amelioration of endothelial dysfunction (Figure 5E). Endothelium-independent relaxations following treatment with sodium nitroprusside were similar between the vehicle and AICAR-treated groups (Figure 5F). In addition, AICAR treatment did not alter the plasma lipid concentrations (Supplemental Table I). Hence,
our findings suggest the possibility that the improvement in the vasodilatory responses in AICAR-treated mice may be brought about by a decreased aortic accumulation of cholesterol and 7-oxysterols.

AMPK Blockade or ABCG1 siRNA Gene Transfer Reverses Vascular Reactivity

To determine whether AMPK antagonists affect vascular reactivity in vivo, we treated HCD-fed C57BL/6J mice with AICAR in the presence or absence of the AMPK antagonist compound C. In AICAR-treated mice administered compound C, there was a significant decrease in ABCG1 expression (Supplemental Figure VA) and eNOS activity (Supplemental Figure VB), accompanied by an increase in ROS (Supplemental Figure VC) and a decrease in the cGMP levels (Supplemental Figure VD). Furthermore, in compound C-treated mice, the improved vasodilator responses to ACh by AICAR were reversed to levels observed in control mice (Supplemental Figure VE).

To further determine the critical role of ABCG1 in the improvement of endothelial dysfunction mediated by AMPK, we suppressed ABCG1 expression using in vivo siRNA technology. In these experiments, wild-type mice were injected at the tail vein with $5 \times 10^9$ plaque-forming units of Ad-ABCG1 siRNA or an empty control vector (Ad). AICAR-mediated upregulation of mouse aortic ABCG1 protein expression was found to be completely abrogated after infection with ABCG1 siRNA, compared with Ad (Figure 6A). Furthermore, in AICAR-treated mice, there was an increase in aortic ROS formation, as measured by lucigenin chemiluminescence, and a decrease in cGMP levels in Ad-ABCG1 siRNA-infected mice compared with Ad-infected mice (24.24 ± 4.47 versus 51.62 ± 2.41 relative light unit (RLU)/second per mg of aorta, $P<0.05$, $n=6$; Figure 6B) and (0.75 ± 0.05 versus 0.39 ± 0.05 pmol/mg protein, $P<0.05$, $n=6$; Figure 6C), respectively. In addition, in AICAR-treated mice, vascular reactivity experiments revealed impaired vasodilation in response to ACh in Ad-ABCG1 siRNA-infected mice, an effect that was not observed in the Ad-infected mice (Figure 6D).

Discussion

The findings of our present study demonstrate for the first time that AMPK activation protects against hypercholester-
olemia-induced endothelial dysfunction via the ABCG1-mediated oxysterol efflux from vascular ECs. The following findings support this conclusion: (1) AMPK induces ABCG1 expression in ECs by regulating ABCG1 mRNA stability via its 3′-UTR; (2) at the cellular level, increased ABCG1 expression by AMPK activation promotes cholesterol and 7-KC efflux to HDL in ECs, causing a marked reduction in oxidative stress, the preservation of eNOS activity, and a concomitant increase in NO bioavailability; (3) in vivo experiments using HCD-fed C57BL/6J mice show that AICAR treatment increases vascular ABCG1 expression, reduces the cholesterol/7KC contents, decreases the ROS levels, and increases the cGMP levels, leading to a significant alleviation of vascular dysfunction; and (4) the protective effects of AICAR are lost in mice treated with the AMPK inhibitor compound C or in those transfected with Ad-ABCG1 siRNA. Hence, our current data indicate that AMPK exerts its protective effects against endothelial dysfunction by inducing ABCG1 expression and thereby promoting cholesterol efflux from ECs.

ABCG1 is the best-characterized cholesterol transporter to date and has been shown to be expressed in vascular ECs.26 In our current study, we demonstrate that AMPK activation by AICAR increases ABCG1 mRNA expression in a dose- and time-dependent fashion in ECs. AICAR treatment was also found to increase ABCG1 protein expression, as well as upregulating the function of ABCG1 by increasing diocetylcarboxyaniniodide-labeled HDL binding in vascular ECs. Previous studies that have addressed the role of AMPK may therefore have yielded equivocal results because of the unique role of AICAR as the means to increase AMPK activity. However, AICAR is regarded as a nonspecific AMPK activator and may affect other AMP-dependent enzymes.27 In our present study, we used metformin and A-769662 as complementary methods of AMPK activation and found comparable effects of these agents on ABCG1 expression in ECs. Because the mechanism underlying metformin- and A-769662-mediated AMPK activation differs from that of AICAR,21,28 we are confident that our findings for the effects of AICAR are indeed due to AMPK activation. This contention is supported by our observations that both genetic and pharmacological approaches to block AICAR-mediated AMPK activation reverse the protective effects of AICAR in vitro and in vivo. To then examine the molecular mechanism by which AICAR regulates ABCG1 expression, we analyzed its impact on the LXR nuclear hormone receptor, which has been shown to be a critical regulator of ABCG1 expression.29 However, we found that AICAR treatment does not stimulate the transcriptional activity of LXR in a reporter assay. AMPK also does not stimulate the transcription of ABCG1. Thus, posttranscriptional regulation appears to be the main mechanism underlying the effects of AMPK on endothelial ABCG1 expression. In the presence of the general inhibitor of gene transcription actinomycin D, we further found that the mRNA half-life of ABCG1 was considerably extended in AICAR-treated cells versus vehicle-treated cells. Therefore, our findings support the conclusion that the 3′-UTR of ABCG1 mRNA contributes to the stabilization of ABCG1 transcripts.

Figure 6. ABCG1 inhibition abolishes the impact of vascular reactivity by AICAR. A, Two groups of C57BL/6J mice received a high-fat, cholesterol-rich diet (HCD) for 12 weeks. The animals were also randomly administered either AICAR (200 mg · kg⁻¹ · d⁻¹) or vehicle (0.9% NaCl). During the final 2 weeks of the 12-week treatment period, the vehicle- and AICAR-treated mice were injected at the tail vein with 5 × 10⁹ plaque-forming units of a recombinant adenovirus containing mouse ABCG1 siRNA under the control of the cytomegalovirus promoter (Ad-ABCG1 siRNA, n = 6) or an empty viral vector (Ad, n = 6), respectively. The animals were subsequently euthanized, and the aortas were excised for analysis. A, At 14 days posttransfection, ABCG1 protein expression was measured by Western blot. B, The aorta superoxide levels were determined by lucigenin chemiluminescence. C, cGMP levels were measured as the indicator of the levels of bioavailable NO. D, Endothelial relaxation was assessed by vascular reactivity to increasing concentrations of ACh. Data represent the mean±SEM; n = 6 per group. *P < 0.05.
duced total cholesterol and 7-KC mass efflux from HAECs and that this was significantly diminished by ABCG1 siRNA. ABCA1, another member of the ATP-binding cassette transporter family, has also been shown to mediate cholesterol and phospholipid efflux to lipid-poor apolipoprotein A-I and could be detected on ECs. However, incubation with ABCA1 siRNA did not affect the sterol efflux induced by AICAR. These results indicate that ABCG1 is necessary to promote the efflux of 7-KC from HAECs induced by AICAR.

The key marker of vascular integrity and endothelial function is the preserved bioavailability of NO, which is regulated by eNOS activity. The NO pathway is impaired in hypercholesterolemia after the breakdown of NO for its reaction with O$_2^-$, which is known to inactivate NO and generate ONOO$^-$. Our current data indicate that the profound improvements in endothelial function induced by AICAR are associated with the upregulation of aortic eNOS activity, leading to enhanced cGMP production. At the same time, we observed a marked decrease in aortic superoxide levels, assessed by L-012 chemiluminescence and dihydroethidium (DHE) staining, in AICAR-treated mice compared with vehicle-treated mice. Hence, it appears that the modulation of eNOS activity by AICAR treatment results in increased NO bioavailability and decreased superoxide production, consistent with the observed improvement in endothelial-dependent vasorelaxation.

Recently, Terasaka et al. have reported that ABCG1 maintains EC function in HCD-fed mice by promoting the efflux of cholesterol and 7-oxysterols, thus highlighting an important role for ABCG1 in preserving endothelial function. However, whether the amelioration of hypercholesterolemia-mediated vascular impairment by AICAR is related to ABCG1 has not yet been explored. To further substantiate our present in vitro findings by in vivo analyses, we examined the effects of AICAR administration on HCD-fed C57BL/6J mice. Notably, we observed that ABCG1 expression is significantly enhanced in aortas from AICAR-treated mice compared with vehicle-treated mice. In accordance with these findings, the total cholesterol (mainly 7-KC) accumulation in aortas was found to be markedly decreased by AICAR treatment compared with vehicle-treated mice. Our studies have thus uncovered a novel mechanism whereby AMPK activation may be involved in an increased efflux of cholesterol and 7-oxysterols via the ABCG1 pathway, thus highlighting an enhanced NO bioavailability and decreased superoxide production, consistent with the observed improvement in endothelial-dependent vasorelaxation. Taken together, our current findings suggest that AMPK activation prevents hypercholesterolemia-mediated endothelial dysfunction and that the underlying mechanism may involve an increased efflux of cholesterol and 7-oxysterols via the ABCG1 pathway. We thus provide evidence that AMPK is potentially a critical target for the future treatment of endothelial dysfunction in vascular disease.

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**Disclosures**

None.

**References**


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AMPK regulates ABCG1-mediated oxysterol efflux from endothelial cells and protects against hypercholesterolemia-induced endothelial dysfunction

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Materials and Methods

Materials. AMPK agonist AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), anti-phospho-AMPK Thr-172 and anti-β-actin were purchased from Cell Signaling Technology. AMPK antagonist compound C was obtained from Calbiochem (San Diego, Calif). GW3965, a synthetic liver X receptor (LXR) agonist, and metformin was purchased from Sigma (Sigma-Aldrich). A-769662, a cell permeable drug that potently activates AMPK in cells, was provided by Symansis Pty Ltd. Dihydroethidine (DHE) was provided from Molecular Probes. Human 3,3′-dioctadecyldiocarbocyanine labeled-fluorescent high density lipoprotein (DiI-HDL) was obtained from Biomedical Technologies.

Plasmid. The luciferase reporter plasmid of ABCG1 (pABCG1 Luc) was constructed as indicated and kindly donated by Sabol SL (Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health).

Adenoviruses. The adenoviral vector expressing a dominant-negative AMPKα mutant (Dn-AMPK) and a constitutively active form of AMPK (Ca-AMPK) were kindly donated as a gift by Dr J. Ha (Department of Molecular Biology, Kyung Hee University, College of Medicine, Seoul, Korea). The cDNA encoding Dn-AMPK or Ca-AMPK was inserted into the EcoRI/Xhol sites of the pAdTrack-CMV shuttle vector. The resulting vector was then electroporated into BJ5138 cells containing the AdEasy adenoviral vector to produce
the recombinant adenoviral plasmid. The recombinant viruses were amplified in HEK293 cells and isolated by cesium chloride density centrifugation. The viruses were collected and desalted, and the titers were measured using Adeno-X™ Rapid titer (BD Bioscience, San Jose, CA) according to the manufacturer’s instructions.²

**Cell Culture.** Human aortic endothelial cells (HAECs) were purchased from Cell Applications Inc. (San Diego, CA) and cultured in M199 medium supplemented with FBS (20% vol/vol), penicillin (100 U/mL), streptomycin (100 μg/mL), heparin (90 μg/mL), and endothelial cell growth supplement (20 μg/mL). The cells were grown at 37°C in humidified 5% CO2 and used for experiments between passages 3 and 5.

Bovine aortic endothelial cells (BAECs, JCRB0155) were obtained from Health Science Research Resources Bank (HSRRB), JAPAN HEALTH SCIENCES FOUNDATION. The cells were cultured in Dulbecco's Modified Eagle Media (DMEM) with 10% fetal calf serum (FCS, Gibco).

**ABCG1 mRNA.** Total RNA was extracted from the cultured cells using TRIzol reagent according to the protocol provided by the manufacturer (Life Technologies). Real-time quantitative PCR analysis was used to measure the relative levels of ABCG1 mRNA expression. The amplification was performed on ABI Prism 7900-HT Sequence Detection System and Universal MasterMix (Applied Biosystems). Levels of the ABCG1 mRNA were subsequently normalized to GAPDH mRNA levels.³,⁴

For Northern blot analysis, 5 μg total RNA was fractionated on 1.2% formaldehyde-agarose gels and transferred onto positively charged nylon membranes (Roche). Hybridization and detection of mRNA was performed using a Digoxigenin Luminescent Detection Kit (Roche) according to the manufacturer’s instructions.

**ABCG1 Protein.** Proteins were size-fractionated electrophoretically using SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with antibody to ABCG1 (#Cat.ab36969, Abcam, Cambridge, MA) and visualized using an enhanced chemiluminescence detection system (ECL, Cell Signaling

²
Anti-β-actin (Cell Signaling Technology Inc) was used to control for equal protein loading.

**ABCG1 mRNA Stability.** For mRNA stability measurements, actinomycin D (Act D, 5 mg/mL) was used to inhibit gene transcription. At the indicated time points after the addition of Act D, cells were harvested and total RNA was extracted. The expression levels of ABCG1 at each time point were measured by Northern blot and quantitative RT-PCR and normalized to the GAPDH levels. The remaining mRNA was determined by comparison with the expression level of the relevant gene at the zero time point (designated 100%) when Act D was added.\(^5\)

**Construction of Human ABCG1 3’Untranslated Region Constructs.** Segments of the human ABCG1 3’ untranslated region (UTR) were PCR-amplified using oligonucleotide primers containing flanking SpeI recognition sequences and human genomic DNA as a template. The PCR products were gel-purified and ligated downstream of the firefly luciferase coding region of the pGL3-Control vector (Promega). The pGL3-Control vector was chosen because it contains the SV40 promoter without enhancers; therefore, changes in luciferase activity can be attributed to the effect of 3’-UTR inserts. The following primers were used to amplify the 3’-UTR of human ABCG1: forward, 5’-GACTAGTGTACAAAATCCGGGCAGAGA-3’; reverse, 5’-GACTAGTGCTTAAGAAGCACGTGGA-3’. All constructs were confirmed by direct sequencing.

**Dil-HDL Binding.** The cultured cells were incubated with different concentrations of AICAR for 24 hours. After then, the cells were incubated with 5 \(\mu\)g/mL Dil-HDL for another 4 hours. The cells were detached using cell removal buffer containing EDTA, washed and resuspended the cells in FACS solution (PBS with 0.5% BSA and 0.02% sodium azide) at a density of \(1 \times 10^6\) cells/mL. The mean fluorescence intensity (MFI) was analyzed by FACS (FACSort, Becton Dickinson).\(^6\)

**Sterol Mass.** The lipid fractions of abdominal aortas, isolated vascular 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.7

**Sterol Mass Efflux.** HAECs were incubated in culture medium plus 5% lipoprotein-deficient serum with cholesterol (5 μg/mL) and 7-KC (5 μg/mL) for 24 hours. The next day, cells were washed with PBS and then incubated in medium plus 5% lipoprotein-deficient serum alone or supplemented with human 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.8

**NOS Activity.** The NO synthesizing activity was determined by quantifying the rate of the conversion of [³H]L-arginine to [³H]L-citrulline with a commercial assay kit obtained from Calbiochem according to the manufacturer’s instructions.

**cGMP Levels.** Confluent HAECs were pretreated with 0.5 mmol/L isobutylmethylxanthine (IBMX) at 37°C for 15 minutes. Cells were then treated with AICAR in the presence of IBMX. The reaction was stopped by the addition of ice-cold 6% trichloroacetic acid and the supernatant fractions of the cellular lysates were extracted, dried and acetylated. Cyclic GMP levels were quantitated using an enzyme immunoassay kit (Cayman) and results standardized by protein levels. For studies performed in isolated aortas, aortas were harvested, rinsed with PBS, and dissected free of adventitia. Cyclic GMP levels were determined in aortic homogenates and standardized to total protein.9

**GSH/GSSG Ratio.** The ratio of glutathione (GSH) to glutathione disulfide (GSSG) was measured using a spectrophotometric GSH/GSSG ratio assay kit (Calbiochem) according to the manufacturer's instructions.
**Gene Silencing by Small Interfering RNA (siRNA).** Complementary RNA (cRNA) oligonucleotides derived from human ABCG1 and ABCA1 sequence (ONTARGETplus SMARTpool targeting human ABCG1 and ABCA1) were obtained from Dharmacon (Chicago, USA) and used to knockdown ABCG1 and ABCA1 expression in HAECs. Scrambled oligonucleotides ONTARGETplus siCONTROL Non-Targeting Pool were used as control. Expression levels of ABCG1 and ABCA1 in transfected cells were determined by real-time PCR and immunobloting.3

To decrease ABCG1 expression in vivo, a recombinant adenovirus encoding mouse ABCG1 siRNA under control of the CMV promoter, Ad-ABCG1 siRNA, or an empty viral vector, Ad, was utilized. To create the recombinant virus, Ad-ABCG1 siRNA was linearized with PacI and transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen). The virus was harvested from the cells by three cycles of freezing and thawing in PBS containing 10% (vol/vol) glycerol. Viral amplification was performed by several rounds of infection of HEK-293 cells and the titer of the Ad-ABCG1 siRNA produced was determined.

**Mice and Procedures.** Animal experiments were conducted in accordance with institutional guidelines of Sun Yat-Sen University. Male C57BL/6J mice (Jackson Laboratories) were used for this study. The animals were maintained in a 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum. All mice were fed a normal chow diet (NCD, n = 12), or high-fat, cholesterol-rich diet (HCD, n = 12) for 12 weeks that contained 21% fat, 19.5% casein, and 1.25% cholesterol (the Research Diets, Inc. formula D12451, with minor modification) starting at 6 wks of age. Animals were randomized to receive either AICAR (n = 6, 200 mg · kg⁻¹ · d⁻¹) or vehicle (n = 6, 0.9% NaCl) via subcutaneous injection once daily. In the compound C group, mice were intraperitoneally injected AICAR (200 mg · kg⁻¹ · d⁻¹) simultaneously with AMPK inhibitor-compound C (20 mg · kg⁻¹ · d⁻¹).

**AMPK Activity.** AMPK activity was measured by monitoring phosphorylation of the SAMS peptide substrate following a previously described protocol.9

5
**Vascular Reactivity.** For endothelial function experiments, aortas were cut into rings (2 to 3 mm long). The rings were attached to a force transducer, suspended in an organ chamber filled with 6 mL of Krebs-Ringer solution (37°C, pH 7.4) and bubbled with 95% O₂, 5% CO₂. Rings were slowly subjected stepwise to a predetermined optimal tension of 30 millinewton (mN) and allowed to equilibrate for at least 30 min. Following equilibration, each ring was precontracted with phenylephrine (PE, 3 μmol/L). Following 60-90 minutes of contraction, a relaxation concentration response curve was generated by adding four cumulative additions of the endothelium dependent vasodilator acetylcholine (Ach, 10⁻⁹ to 10⁻⁵ mol/L) every 3 minutes. In addition, sodium nitroprusside (SNP, 10⁻⁹ to 10⁻⁵ mol/L, endothelium-independent NO-releasing agent) was added into the organ bath, and endothelium-independent vasorelaxation was recorded.¹⁰,¹¹

**Vascular Superoxide Production.** Superoxide release in aortic segments was determined by [8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H)dione] (L-012, Wako Chemicals) chemiluminescence. Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer (pH 7.4; NaCl 99.0 mmol/L, KCl 4.7 mmol/L, CaCl₂ 1.87 mmol/L, MgSO₄ 1.20 mmol/L, sodium HEPES 20.0 mmol/L, K₂HPO₄ 1.03 mmol/L, NaHCO₃ 25.0 mmol/L, D(+)Glucose 11.1 mmol/L). Connective tissue was removed and aortas were cut into 2-mm segments. The aortic segments were transferred into scintillation vials containing Krebs-HEPES buffer with L-012 (100 μmol/L) and were incubated for 15 minutes. Chemiluminescence was then assessed over 15 minutes in a scintillation counter (Lumat LB 9501, Berthold, Bad Wildbad, Germany) in 1 minute intervals. The vessel segments were then dried and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per mg aortic tissue.¹²

As a second measure of ROS accumulation, unfixed aortas were immediately frozen in OCT and cut into 10 μm-thick sections and placed on a glass slide. The sections were incubated with 20 μmol/L dihydroethididine (DHE, Molecular Probes) in a dark humidified chamber at 37°C for 30 minutes. In situ production of superoxide was visualized by fluorescence microscopy. Paired aortas from active and inactive mice were processed in
parallel, and images were acquired with identical acquisition parameters and were stored digitally.\textsuperscript{13}

\textit{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 minutes. 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 antibody (Millipore) at 4°C for 15 minutes 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.\textsuperscript{14}

\textit{Statistical analysis.} Continuous data were expressed as mean ± SEM. Comparison between groups was performed by Student’s paired two-tailed t-test. Two-way analysis of variance (ANOVA) was used to examine differences in response to treatments between groups, with post-hoc analysis performed by the method of Student-Newman-Keuls. $P<0.05$ was considered significant.

\textbf{References}


AMPK regulates ABCG1-mediated oxysterol efflux from endothelial cells and protects against hypercholesterolemia-induced endothelial dysfunction

Dan Li, Yuan Zhang, Jing Ma, Wenhua Ling, Min Xia

Online Figure I. Induction of ABCG1 expression by AMPK activation.

(A-B) HAECs were treated with Metformin (1 mmol/L) or A769662 (100 μ mol/L) for 8 hours. (A) ABCG1 mRNA and (B) protein expression were measured by quantitative RT-PCR and Western blot.

(C) HAECs were infected with adenoviruses encoding constitutively active (Ca) or dominant negative (Dn) forms of AMPK α in presence of AICAR (1 mmol/L) or vehicle. Forty microgram of total protein were used for western blot.

(D-E) BAECs were treated with vehicle or AICAR (0.25 mmol/L or 2 mmol/L) for 8 hours. Total RNA was extracted and ABCG1 gene expression were analyzed by quantitative RT-PCR and Northern blot.

(F) HAECs were pretreated AMPK inhibitor compound C (10 mmol/L) for 2 hours or transfected with Dn-AMPK α. After then the cells were incubated with AICAR (2 mmol/L) for another 24 hours. The uptake of Dil-HDL was measured by FACScan with $1 \times 10^5$ cells per sample. The mean fluorescence intensity (MFI) of untreated cells is expressed as 100%. The data shown are expressed as percent of control from three independent measurements.
Online Figure II. RNA interference mediates inhibition of ABCG1 and ABCA1 expression.
HAECs were transfected with ONTARGETplus SMARTpool targeting human (A) ABCG1 and (B) ABCA1. Scrambled oligonucleotides ONTARGETplus siCONTROL were used as control. After then, Western blot analyses using antibodies against ABCG1, ABCA1 and actin were performed to test transfection efficiency. Representative blots from 3 independent assays are presented.

Online Figure III. AICAR treatment improves endothelium-dependent vasorelaxation.
C57BL/6J mice fed NCD and HCD was treated with AICAR (200 mg · kg⁻¹ · d⁻¹, n = 12) or vehicle (0.9% NaCl, n = 12) for 12 weeks. (A) AMPK phosphorylation in tissue extracts from aorta was analyzed by Western blot. The data represent results of three independent experiments. (B) AMPK kinase activity assays with the use of SAMS peptide as a substrate were also performed for extracts from aorta. (C) Representative sections of dihydroethidium staining for superoxide in aortic sections. Red staining represents 2-hydroxyethidium fluorescence and superoxide localization, and blue staining represents autofluorescence in elastin layers in the medial wall. (D) Levels of bioavailable NO were assessed by measuring cGMP levels. Data represent means ± SEM. n = 6 per group. *P<0.05 vs vehicle.
Online Figure IV. AICAR induces ABCG1 expression and reduces cholesterol/7-KC accumulation in aortic ECs from HCD-fed mice.

(A) C57BL/6J mice were put on a HCD for 12 weeks in the presence of AICAR (200 mg · kg⁻¹ · d⁻¹) or vehicle. After treatment, aortic ECs fractions and ABCG1 mRNA expression were measured by Real-time quantitative RT-PCR. The abundance of ABCG1 mRNA was expressed as arbitrary unit. *P<0.05 compared to vehicle. (B) ABCG1 protein expression was assayed by Western blot. The representative blots were shown. (C) Cholesterol contents and (D) 7-KC accumulation in the ECs fractions were determined. The results are represented as means ± SD of 6 mice. *P<0.05 vs vehicle.

Online Figure V. AMPK antagonism inhibits improvement of vascular reactivity by AICAR.

(A) C57BL/6J mice were treated with AICAR (n = 6, 200 mg · kg⁻¹ · d⁻¹) or vehicle (n = 6, 0.9% NaCl) in the absence or presence of compound C (n = 6, 20 mg · kg⁻¹ · d⁻¹). ABCG1 protein expression was determined by Western blot from three independent experiments. (B) eNOS activity was determined as indicated in Methods. (C) Superoxide production in aorta was analyzed by L-012 chemiluminescence. (D) cGMP levels were measured as an indicator of bioactive NO. (E) The vascular reactivity to increasing concentrations of acetylcholine (Ach) was examined in vehicle-, AICAR- and AICAR plus compound C-infused C57BL/6J mice (n = 6 per treatment group). Data represent mean ± SEM. *P<0.05 between AICAR and AICAR + compound C.
Online Figure I

**A**

![Graph showing ABCG1 mRNA expression](image)

**B**

![Image of Western blots showing ABCG1 and β-actin](image)

**C**

![Image of Western blots showing ABCG1 and β-actin](image)

**D**

![Graph showing ABCG1 mRNA expression](image)
Online Figure II

A

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Control</th>
<th>ABCG1</th>
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B

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ABCG1

β -actin
Online Figure III

A

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<tr>
<th>AICAR</th>
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<tr>
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<td></td>
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phos-AMPK

total AMPK

B

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<tr>
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<td>HCD+AICAR</td>
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C

<table>
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<tr>
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<td>AICAR</td>
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<td>HCD</td>
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D

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<th>cGMP levels (pmol/mg protein)</th>
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<td>HCD</td>
<td></td>
</tr>
</tbody>
</table>
Online Figure IV

A

ABCG1 mRNA expression (arbitrary unit)

Vehicle  | AICAR
---------|---------
4000     | 12000   *

B

Vehicle  | AICAR
---------|---------
ABCG1    | β-actin

C

Cholesterol mass (μg/mg protein)

Vehicle  | AICAR
---------|---------
100       | 60 *    

D

7-KC (μg/mg protein)

Vehicle  | AICAR
---------|---------
4         | 2 *     
Online Figure V

A

ABC1G

β-actin

AICAR compound C

- + +

- - +

B

eNOS activity (% Control)

Control AICAR AICAR-Compound C

0 50 100 150 200 250 300

C

L012 chemiluminescence (RFU/mg aorta)

Control AICAR AICAR-Compound C

0 500 1000 1500 2000 2500

D

cGMP levels (pmol/mg protein)

Control AICAR AICAR-Compound C

0 0.2 0.4 0.6 0.8 1
**Online Table I. Plasma lipid profiles of C57BL/6J mice treated with or without AICAR**

<table>
<thead>
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<th>Variables</th>
<th>NCD</th>
<th>HCD</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>AICAR</td>
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<tr>
<td>Total cholesterol</td>
<td>3.14 ± 0.13</td>
<td>3.25 ± 0.28</td>
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<tr>
<td>Triglycerides</td>
<td>1.34 ± 0.45</td>
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<tr>
<td>Free fatty acid</td>
<td>0.55 ± 0.14</td>
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Data are means ± SEM of 12 mice. *$P<0.05$ vs NCD-fed vehicle and AICAR.