Hypercholesterolemia Suppresses Inwardly Rectifying K+ Channels in Aortic Endothelium In Vitro and In Vivo

Yun Fang, Emile R. Mohler III, Esther Hsieh, Hashim Osman, Seyed M. Hashemi, Peter F. Davies, George H. Rothblat, Robert L. Wilensky, Irena Levitan

Abstract—Inwardly rectifying K+ (Kir) channels are responsible for maintaining endothelial membrane potential and play a key role in endothelium-dependent vasorelaxation. In this study, we show that endothelial Kir channels are suppressed by hypercholesterolemic levels of lipoproteins in vitro and by serum hypercholesterolemia in vivo. Specifically, exposing human aortic endothelial cells to acetylated low-density lipoprotein or very low density lipoprotein resulted in a time- and concentration-dependent decrease in Kir current that correlated with the degree of cholesterol loading. The suppression was fully reversible by cholesterol depletion. Furthermore, a decrease in Kir current resulted in depolarization of endothelial membrane potential. Most important, the flow sensitivity of Kir currents was also impaired by cholesterol loading. Specifically, flow-induced increase in Kir current was suppressed by 70%, and flow-induced hyperpolarization was almost completely abrogated. Furthermore, we show that hypercholesterolemia in vivo also strongly suppresses endothelial Kir currents and causes a shift in endothelial membrane potential, as determined by comparing the currents in aortic endothelial cells freshly isolated from healthy or hypercholesterolemic pigs. Therefore, we suggest that suppression of Kir current is one of the important factors in hypercholesterolemia-induced endothelial dysfunction. (Circ Res. 2006;98:1064-1071.)

Key Words: K channels • cholesterol • lipoproteins • flow • vasodilatation

Numerous studies have shown that a major risk factor for the development of cardiovascular disease is plasma hypercholesterolemia, elevation of low- and very low-density lipoproteins (LDL and VLDL), as well as the oxidized modification of LDL, oxidized LDL (oxLDL).1,2 Endothelial dysfunction develops in the early stages of cardiovascular disease and is a strong predictor of its development.3 A hallmark of endothelial dysfunction in vivo is impairment of flow-mediated vasodilatation (FMV). Indeed, several studies have shown that plasma hypercholesterolemia impairs FMV both in humans and in animal models of atherosclerosis.4,5 However, molecular mechanisms that underlie hypercholesterolemia-induced endothelial dysfunction are poorly understood.

Our studies focus on the impact of hypercholesterolemia on endothelial inwardly rectifying K+ (Kir) channels, the major determinants of endothelial membrane potential6,7 and putative flow sensors.8–10 The resting membrane potential in endothelial cells ranges between approximately −30 and −80 mV depending on a specific endothelial cell type.7 Aortic endothelial cells typically have resting potentials in a strongly hyperpolarizing range (approximately −60 mV/−80 mV) both in culture8,11–13 and in intact aortas,14 consistent with a major contribution of K+ channels. Several types of K+ channels are expressed in endothelial cells: Kir channels that maintain stable membrane potential under resting condition and are responsible for flow-induced hyperpolarization and several types of Ca2+-activated K+ (Kca) channels responsible for agonist-induced hyperpolarization.6,7 Indeed, blocking endothelial Kir channels by Ba2+, a known blocker of Kir but not of Kca, results in significant depolarization15 and inhibits flow-induced vasorelaxation in cerebral arteries,16 consistent with the proposed role of Kir channels in flow-mediated vasorelaxation.17 Our recent study has shown that K+ conductance in human aortic endothelial cells (HAECs) is underlied mainly by the two members of Kir2 subfamily of inward rectifiers: Kir2.1 and Kir 2.2.10 We have also shown that enriching bovine aortic endothelial cells with cholesterol using methyl-β-cyclodextrin (MβCD) strongly suppresses Kir current in these cells18 and that Kir channels are suppressed by MβCD-induced cholesterol enrichment when heterologously expressed in a null cell line.19

In this study, we demonstrate that endothelial Kir is strongly suppressed by acetylated LDL (acLDL) and VLDL in vitro and by plasma hypercholesterolemia in vivo, which, in turn, results in membrane depolarization. Furthermore, we show that atherogenic lipoproteins impair the flow sensitivity of the current and virtually abolish flow-induced membrane...
hyperpolarization. Also, consistent with the suppression of flow-sensitive Kir channels, we show that plasma hypercholesterolemia significantly suppresses flow-induced vasodilation in vivo. Therefore, we suggest that sensitivity of Kir channels to membrane cholesterol may be an important mechanism underlying hypercholesterolemia-induced endothelial dysfunction and impairment of flow-induced vasorelaxation.

Materials and Methods

Cells
HAECS (BioWhittaker Cambrex) were maintained between passages 3 and 5 in endothelium growth medium-2 (EGM-2; Cambrex) containing 2% FBS.10

Lipoprotein Treatments and Assessment of Cellular Cholesterol
Human acLDL (2 mg/mL) and oxLDL (2 mg/mL; 10 to 15 nmol/mg protein thiobarbituric acid reactive substances [TBARS]) were purchased from Biomedicals Technologies Inc.; human VLDL (1.36 mg/mL) was purchased from Biodesign International. For treatment, acLDL was diluted in EGM-2 media with 0.2% FBS, and VLDL was diluted in EGM-2 with lipoprotein-deficient serum. Cells were preincubated in lipoprotein-deficient serum for 15 hours before an experiment. Cholesterol-loaded HAECS were depleted of cholesterol with MβCD.20 Cellular cholesterol in HAECS was measured by Amplex Red cholesterol assay kit (Molecular Probes) according to manufacturer specifications. Cellular cholesterol in freshly isolated porcine aortic endothelial cells (PAECs) was measured by gas-liquid chromatography.20 The two methods were compared in cultured cells and yield identical results (data not shown).

Electrophysiology
Ionic currents and membrane potentials of endothelial cells were measured using standard voltage- and current-clamp techniques, as described previously. Whole-cell currents were recorded during 500-ms linear voltage ramps from −160 to +60 mV at an interpulse interval of 3 s. The external solution for voltage-clamp experiments contained (in mmol/L): 156 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, and 1 EGTA, pH 7.3. The standard external solution for the current-clamp experiments contained (in mmol/L): 152 NaCl, 4 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, and 1 EGTA, pH 7.3. The pipette solution for the current-clamp experiments contained (in mmol/L): 152 NaCl, 4 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, and 1 EGTA, pH 7.3. The pipette was saturated with cholesterol and had no significant effect (Figure 1A). In all experimental conditions, HAECS showed pronounced strongly rectifying Kir current, consistent with our previous study.10 Typical Kir currents recorded from HAECs, which were exposed to 5 mg/mL acLDL, respectively, when compared with controls were compared in cultured cells and yield identical results (data not shown).

Electrophysiology

Electrophysiology

Electrophysiology

Suppression of Endothelial Kir by Hypercholesterolemia In Vitro

Suppression of Kir by AcLDL
To determine the effect of atherogenic lipoproteins on endothelial Kir current, HAECS were exposed to acLDL, which is widely used in lipoprotein research to load cells with cholesterol.24,25 As expected, exposure to acLDL resulted in a significant increase in the level of free cholesterol of HAECS, and the effect was reversible by treating the cells with MβCD (Figure 1A). In all experimental conditions, HAECS showed pronounced strongly rectifying Kir current, consistent with our previous study.10 Typical Kir currents recorded from control cells, which were exposed to 5 mg/mL acLDL, and cells preloaded with cholesterol and then depleted were shown in Figure 1B. The mean current density decreased by 20% and 52% in cells exposed to 5 mg/mL or 50 mg/mL acLDL, respectively, when compared with controls (Figure 1Bii). Furthermore, exposing acLDL-preloaded cells to 5 mmol/L MβCD resulted in rescue of the Kir current, whereas exposing the cells to the same concentration of MβCD saturated with cholesterol had no significant effect (Figure 1Bii). These observations suggest that MβCD-induced rescue of Kir is attributable to removing the surplus of cholesterol. We also show here that although Kir currents are significantly lower in physiological K+ concentration than in high K+, the effect of hypercholesterolemia is similar under both conditions (Figure 1C) and is not attributable to changes in Kir rectification property (supplemental Figure I, available online at http://circres.ahajournals.org), consistent with our previous observations in cholesterol-loaded bovine endothelium.18

AcLDL-induced suppression of endothelial Kir developed gradually with time, and there was a clear correlation between
suppression of the current and an increase in cellular cholesterol (supplemental Figure II). Enrichment of HAECs with cholesterol using MβCD–cholesterol complex also suppressed Kir (data not shown), as described previously in bovine endothelium. Thus, exposure to acLDL resulted in significant suppression of Kir current in HAECs, whereas depletion of cholesterol from acLDL-pretreated cells fully restored the current.

**Suppression of Kir by VLDL**

To further test whether endothelial Kir current is suppressed by physiological cholesterol carriers, the cells were exposed to VLDL, one of the most common atherogenic lipoproteins associated with hypercholesterolemia-associated endothelial dysfunction and cardiovascular diseases. Consistent with the previous studies in rabbit aortic endothelial cells, exposure of HAECs to 5 μg/mL or 50 μg/mL human VLDL for 24 hours resulted in a significant increase in the level of cellular cholesterol (Figure 2A). Furthermore, similar to the effect of acLDL described above, HAECs exposed to VLDL also demonstrated significant suppression of the endothelial Kir current (Figure 2B and 2C). In this case, both the degree of cholesterol loading and the degree of current suppression were similar in cells exposed to 5 μg/mL and to 50 μg/mL VLDL. As described above for acLDL, exposing VLDL-pretreated cells to MβCD resulted in a decrease of cellular cholesterol and rescue of endothelial Kir currents (Figure 2A and 2B). Also, VLDL-induced suppression was similar in high- and in low-K⁺ solutions (Figure 2C). The similarity between the ability of MβCD to rescue Kir currents in acLDL- and in VLDL-pretreated cells suggests that both types of lipoproteins suppress endothelial Kir current by similar mechanisms.

**OxLDL Has No Effect on Endothelial Kir**

In contrast to acLDL and VLDL, exposure of HAECs to oxLDL does not affect the level of cellular cholesterol nor Kir current (Figure 3). Cells were exposed to 10 to 50 μg/mL oxLDL, similar to the circulating levels of oxLDL in human plasma (7 to 35 μg/mL). The oxidation state of oxLDL was 10 to 15 nmol/mg protein TBAR, as in previous studies. Figure 3A shows the levels of free cholesterol and Kir currents in cells exposed to 50 μg/mL oxLDL for 24 hours. Similar results were observed at shorter exposures.

**VLDL Impairs the Flow Sensitivity of Kir in HAECs**

Consistent with previous studies, Kir currents in HAECs are sensitive to flow (supplemental Figure III). A statistically significant flow-induced increase in the current amplitude was observed (n=8 cells), which was fully reversible and sensitive to repetitive flow applications. Flow sensitivity of Kir was determined at 2 dyne/cm² shear stress, the level that is above half-maximal activation of the current (≈0.7 dyne/cm²). The values of reversal potentials were measured for each trace, and only cells that had no flow-induced increase in nonspecific leak were analyzed. The amplitude of the shear stress–induced component of the Kir current (KirSS) was calculated as the difference between the current amplitudes under flow and under static conditions (representative traces are shown in Figure 4A). Application of 2 dyne/cm² shear stress under normal cholesterol conditions increased Kir amplitude by ≈11% (KirSS/Kirstatic), in agreement with previous reported values of KirSS. Here, we demonstrated for the first time that exposure of HAECs to 50 μg/mL VLDL for 24 hours resulted in significant decrease in KirSS. Application
of 2 dyne/cm² shear stress under the hypercholesterolemic conditions resulted only in ~4% increase of the current (Figure 4B). These observations suggest that atherogenic VLDL specifically suppresses the KirSS in HAECs.

**VLDL Impairs Flow-Induced Hyperpolarization**

The major role of Kir channels in a variety of cells including endothelial cells is to maintain the membrane potential. Therefore, we determined whether hypercholesterolemia-induced suppression of Kir affected the membrane potential and flow-induced membrane hyperpolarization. Figure 5A shows typical recordings of membrane potential under static and flow conditions in control cells and in cells exposed to VLDL. The values of the membrane potential in control cells were significantly more negative than those in cells exposed to VLDL. Under static conditions.
Suppression of Endothelial Kir Current by Hypercholesterolemia In Vivo

Hypercholesterolemia Inhibits FMV
To establish whether hypercholesterolemia resulted in suppression of endothelial Kir in vivo, we analyzed the currents in freshly isolated aortic endothelium in a swine model of dietary induced atherosclerosis. As expected, high-cholesterol diet induced a significant increase in the levels of total plasma and LDL cholesterol, as well as an increase in the high-density lipoprotein levels (Table). Importantly, high-cholesterol diet also resulted in a partial loss of FMV, a marker of endothelial dysfunction, as estimated using ultrasound imaging. %FMV was compared before and four weeks after the initiation of the diet. As shown in the Table, a loss of FMV was observed in the hypercholesterolemic group (n=4; P<0.05), whereas in the control group, no significant change was observed (n=4; P=0.19; NS).

High-Cholesterol Diet Increases the Level of Swine Endothelial Cellular Cholesterol In Vivo
Although multiple studies have shown that serum hypercholesterolemia in vivo increases the level of cellular cholesterol in macrophages and smooth muscle cells, there is little information about the in vivo levels of cellular cholesterol in vascular endothelium. Here, we show that the level of cellular cholesterol in PAECs isolated from hypercholesterolemic pigs was significantly higher than that in control animals (Figure 6B). The level of cellular free cholesterol in freshly isolated pig aortic endothelium is similar to that in aortic endothelium maintained in culture.20,27,28

Hypercholesterolemia Induces Suppression of Kir and Membrane Depolarization
The properties of endothelial Kir and the values of the membrane potentials were compared in PAECs freshly isolated from hypercholesterolemic animals to that observed in HAECs. However, cells isolated from control pigs exhibited strong Kir current (Figure 7A) similar to that observed in HAECs. Although multiple studies have shown that serum hypercholesterolemia in vivo increases the level of cellular cholesterol in macrophages and smooth muscle cells, there is little information about the in vivo levels of cellular cholesterol in vascular endothelium. Here, we show that the level of cellular cholesterol in PAECs isolated from hypercholesterolemic pigs was significantly higher than that in control animals (Figure 6B). The level of cellular free cholesterol in freshly isolated pig aortic endothelium is similar to that in aortic endothelium maintained in culture.20,27,28

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<table>
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<th>Parameter</th>
<th>Total cholesterol</th>
<th>LDL</th>
<th>HDL</th>
<th>%FMV&lt;sub&gt;4 weeks&lt;/sub&gt;</th>
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<td>95±13</td>
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HDL indicates high-density lipoprotein.
Discussion

Our previous studies have shown that Kir is sensitive to the level of cellular cholesterol, suggesting that plasma hypercholesterolemia may impair endothelial Kir and consequently lead to endothelial dysfunction. In this study, we used a combination of in vitro and ex vivo approaches to test the impact of hypercholesterolemic levels of lipoproteins on endothelial Kir current. Our main findings are: (1) endothelial Kir is strongly suppressed by acLDL and by VLDL but not by oxLDL; (2) the degree of current suppression correlates with cellular cholesterol load, and removal of cholesterol rescues the current; (3) lipoprotein-induced cholesterol loading strongly inhibits the flow sensitivity of the current and nearly abolishes flow-induced hyperpolarization; and finally, (4) inducing plasma hypercholesterolemia in a pig model results in Kir suppression and a shift in endothelial membrane potential, which correlates with the suppression of flow-induced vasodilatation in vivo. Together, these observations demonstrate that hypercholesterolemia has a major impact on the dominant membrane conductance and the value of membrane potential in aortic endothelium.

Sensitivity of Kir to Different LDL Species

In Vitro

The in vitro studies were performed in low-passage HAECs shown previously to express significant Kir current similar to that in freshly isolated aortic endothelium, indicating that HAECs represent a suitable model to study endothelial Kir.

Three types of human LDL were used in this study to assess the impact of hypercholesterolemia on endothelial Kir. First, cells were exposed to acLDL, which, although not physiological, is frequently used to test the effect of cholesterol loading on cell function. Exposure of HAECs to acLDL resulted in an ∼2-fold increase in the level of cellular free cholesterol. Importantly, the degree of cholesterol enrichment in acLDL-treated cells was similar to that observed in HAECs exposed to hypercholesterolemic levels (240 mg/dL) of LDL. AcLDL induced suppression of endothelial Kir, and moreover, the rescue of the current by the removal of cholesterol excess provides the first evidence that the current is suppressed by lipoprotein-dependent cholesterol loading.

To verify further whether endothelial Kir is sensitive to physiological cholesterol carriers, HAECs were exposed to VLDL, the second major cholesterol carrier in plasma. Although the total levels of VLDL in plasma are significantly lower than those of LDL, VLDL levels are also known to increase significantly in hypercholesterolemic patients, and...
an increase in the plasma level of VLDL is a significant positive predictor of coronary events.\textsuperscript{26} It is also associated with hypercholesterolemia-induced endothelial dysfunction such as loss of anti-inflammatory and anticoagulant properties and impairment of endothelium-dependent vasorelaxation.\textsuperscript{33,35}

Finally, we also tested whether endothelial Kir is also affected by exposing the cells to oxLDL, an oxidized modification of LDL that is considered to be one of the major risk factors for the development of atherosclerosis and is known to cause endothelial dysfunction.\textsuperscript{1,30} In terms of its effect on cellular cholesterol, oxLDL is predominantly associated with an increase in cellular cholesterol in macrophages, initiating formation of foam cells,\textsuperscript{3} but its effect on endothelial cholesterol is still controversial.\textsuperscript{30} In this study, exposing HAECS to oxLDL had no significant effect on the level of cellular cholesterol and no effect on endothelial Kir. In contrast to a widely accepted notion that acLDL is similar to oxLDL in terms of effects on cellular cholesterol and function, their effects on endothelial cells appear different because acLDL simulates the effects of hypercholesterolemic levels of LDL but not of oxLDL. Our observations suggest that it is LDL and VLDL but not oxLDL that impair endothelial Kir.

**Mechanism of Lipoprotein-Induced Kir Suppression**

Two lines of evidence suggest that lipoprotein-induced suppression of Kir is attributable to an increase in cellular cholesterol level. One is the similarity between acLDL/VLDL-induced Kir suppression and suppression of the current induced by cholesterol enrichment using an artificial cholesterol carrier.\textsuperscript{18} There is also a general correlation between the degree of Kir suppression and cholesterol enrichment across multiple conditions, such as exposing the cells to different LDL modifications, concentrations, and exposure durations. This is consistent with our previous study showing that cholesterol enrichment has a gradual effect on heterologously expressed Kir channels.\textsuperscript{39} Furthermore, full recovery of the current by the removal of cholesterol excess suggests that cholesterol loading is the mediator of the observed effects. However, the mechanisms by which cellular cholesterol regulates channel function is unknown. Sensitivity to cholesterol has been demonstrated for a variety of ion channels,\textsuperscript{36,37} and there is a growing amount of evidence that cholesterol sensitivity of the channels is associated with their partition into cholesterol-rich lipid rafts,\textsuperscript{19,36,37} but molecular details of the regulation are still unclear.

**Sensitivity of Kir to Serum Hypercholesterolemia in a Pig Model**

There are several types of animal models for atherosclerosis, including pigs, rabbits, and genetically engineered mice. We chose the pig because porcine lipoprotein profiles are similar to those in humans\textsuperscript{38} and because hypercholesterolemic pigs develop atherosclerotic lesions within a few months after the initiation of a high-cholesterol diet.\textsuperscript{39} To the best of our knowledge, our study is the first to demonstrate that high-cholesterol diet also significantly increases the cellular level of free cholesterol in porcine aortic endothelium and that the increase is similar to that reported for smooth muscle cells.\textsuperscript{40} Most important, the demonstration that cells freshly isolated from hypercholesterolemic animals have significantly smaller Kir than cells isolated from healthy animals unambiguously demonstrates that hypercholesterolemia-induced Kir suppression occurs in vivo and suggests that it may be an important factor in the development of endothelial dysfunction.

**Physiological Significance of Endothelial Kir Suppression**

A direct consequence of Kir suppression is membrane depolarization, which, in turn, may affect multiple cellular functions. Membrane depolarization was shown to affect Ca\textsuperscript{2+} signaling and release of vaso dilators, NO, and prostacyclin,\textsuperscript{6,7} impair endothelium-dependent vasodilatation,\textsuperscript{41} enhance platelet aggregation,\textsuperscript{42} and to induce the generation of reactive oxygen species.\textsuperscript{43} Membrane depolarization of endothelial cells is also described in several pathological conditions, such as aortic endothelium of stroke-prone spontaneously hypertensive rats,\textsuperscript{44} coronary endothelium of hyperactive rats,\textsuperscript{45} and aortic and lung microvascular endothelia subjected to ischemia.\textsuperscript{46} This study is the first to describe endothelial membrane depolarization caused by hypercholesterolemic conditions. Furthermore, inhibition of the flow sensitivity of Kir and virtual abrogation of flow-induced hyperpolarization suggest that hypercholesterolemia-induced suppression of Kir may play an important role in the impairment of FMV.

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Supplemental Figure 1. Insensitivity of rectifying properties of Kir currents to acLDL. A: Typical Kir current traces recorded from individual control and acLDL-treated cells. A two-pulse voltage protocol was used: 500-ms voltage steps from –160 to +10 mV with increments of 10 mV and immediately followed by 10-ms test pulses to –160 mV. The experiments were performed at 4 mM extracellular K+. B: Normalized current-voltage relationships of Kir currents in control cells (⊙) and in cells treated with 50µg/ml acLDL for 24 hours (▲). Each data set is expressed as mean ± SEM (n= 4-6).

Supplemental Figure 2. Time- and concentration-dependent inhibition of endogenous Kir current in HAECs by acLDL. A: Time-dependent enrichment of cellular cholesterol by acLDL in HAECs. Cells were treated with 50µg/ml acLDL for 1, 6, 12, or 24 hours followed by measurements of cellular cholesterol. B: Time- and dose-curves of the inhibition of endothelial IK current by acLDL. HAECs were exposed to 5µg/ml or 50µg/ml acLDL for 1, 6, 12, or 24 hours, followed by electrophysiological recordings. Each data set is expressed as mean ± SEM.

Supplemental Figure 3. Shear stress-sensitivity of Kir current in HAECs. A: Kir current traces recorded before and under application of a laminar shear stress of 2 dyne/cm² in the same cell. B: The time course of flow-induced modulation of IK current. Voltage ramps were applied with intervals of 3s for the duration of the experiment. The amplitudes of the Kir currents at -160 mV are plotted as a function of time. The bars indicate the durations of the flow. C: Average amplitude of shear stress-induced IK
current in HAECs. Average current amplitudes measured during the application of the flow or after flow cessation were normalized to the current amplitudes prior to the application of the flow in the same cell. Data are Means + SEM (n=5).
Online Supplement  

Y Fang, et al. Atherogenic lipoproteins suppress endothelial Kir

Supplemental Figure 1
A

**Supplemental Figure 3**

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)