Homocysteine Induces 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in Vascular Endothelial Cells
A Mechanism for Development of Atherosclerosis?

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Background—It has been established that hyperhomocyst(e)inemia (HHCy) is an independent and graded risk factor for atherosclerosis, although the molecular link to the atherosclerotic process remains obscure.

Methods and Results—Screening human umbilical vein endothelial cells (HUVECs) with complementary DNA microarray for the gene expression modified by homocysteine (Hcy) revealed that 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) was upregulated. This effect was confirmed using quantitative reverse transcriptase–polymerase chain reaction. Actinomycin D studies revealed that Hcy stabilized HMGCR mRNA ($\tau_{1/2}$, 9.5±1.0 versus 5.0±0.2 hours). Expression of immunodetectable HMGCR in both HUVECs and renal microvascular endothelial cells was increased in Hcy-treated cells in association with the increased abundance of caveolin. Application of a cell-permeable superoxide dismutase mimetic, Mn-TBAP, reversed the Hcy-induced expression of HMGCR. Additional biochemical analysis of the abundance of total cellular cholesterol showed that 0, 20, 50, and 100 $\mu$mol/L Hcy resulted in 22.2±7.3%, 39.5±1.2%, and 50.4±6.8% increase, respectively. Gas chromatography mass spectrometry analysis of extracted cholesterol from Hcy-treated HUVECs and from the culture medium showed 17.8±5.2% and 24.0±14.5% increases, respectively. Application of simvastatin to Hcy-treated cells reduced cellular cholesterol and prevented Hcy-induced suppression of NO production by HUVECs in a dose-dependent manner.

Conclusions—Using a cDNA microarray, the data disclosed an unexpected link between Hcy and cholesterol dysregulation based on the finding of increased abundance of HMGCR mRNA and protein in endothelial cells, demonstrated the possible role of Hcy-induced oxidative stress in this response, and revealed the improvement of endothelial NO production in Hcy-treated HUVECs by statins. Collectively, these findings may provide a solid explanation for the observed proatherogenic effect of HHcy. (Circulation. 2002;105:1037-1043.)

Key Words: endothelium ■ cholesterol ■ statins ■ nitric oxide

Chronic renal failure (CRF) is associated with a 20-fold increased risk of cardiovascular mortality attributable in part to the accelerated atherosclerosis. Hyperhomocyst(e)inemia (HHCy) is common in patients with CRF. It has been established that HHCy is an independent and graded risk factor for development of cardiovascular disease such as stroke, myocardial infarction, peripheral vascular disease, and atherosclerosis.1–3 Endothelial dysfunction is believed to be an important mechanism, initiating the pathogenesis of atherosclerosis.4 Disruption of a delicate balance of the NO system, especially by the increased vascular production of reactive oxygen species (ROS), promotes the development of endothelial dysfunction in HHCy.5–8 However, molecular mechanisms responsible for homocysteine (Hcy)-induced proatherogenic changes in vascular endothelium are not fully understood.

DNA microarray technology has been successfully used in large-scale gene expression analyses of various pathologic conditions, including effects of some proatherogenic agents on endothelial cells.9–12 This approach may provide clues to understanding the complex pathways and their interactions in gene regulation under different conditions. Therefore, we examined gene expression using a cDNA microarray technology in endothelial cells subjected to elevated homocysteine level. The results showed an enhanced expression of mRNA encoding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthesis.

Werstuck et al13 demonstrated that HHCy-induced endoplasmic reticulum (ER) stress in hepatocytes results in an enhanced lipid biosynthesis by activation of the sterol regulatory element binding protein, thus leading to hepatic steato-
Cholesterol homeostasis is regulated through the sterol metabolism and fluxes, which involve HMG-CoA synthase and reductase. Cholesterol increases the abundance of caveolin-1, thus accelerating cholesterol traffic and efflux. The consequences for eNOS function of this cholesterol-induced increase in caveolin abundance is a marked decline in basal NO release. HMGCR inhibitors (statins) have been shown to decrease cardiovascular morbidity and mortality, attributed to reduction of serum lipid levels. Lately, it has been appreciated that statins can restore endothelial function by activating eNOS through Akt signaling and inhibition of the mevalonate-dependent geranylgeranylation of Rho GTPase proteins, independent of their lipid-lowering effects.

On the basis of cDNA microarray detection of induced expression of HMGCR in human endothelial cells, we provide results of experimental findings demonstrating that statins possess an additional effect, amelioration of endothelial dysfunction induced by elevated levels of homocysteine, and present the rationale for the use of statins in HHcy. Collectively, these novel findings may provide a solid explanation for the observed proatherogenic effect of HHcy.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and renal microvascular endothelial cells (RMVECs) were cultured in EBM-2 media (Clonetics). HUVEC was used between passages 3 through 8. At confluence, 50 μmol/L DL-homocysteine (Fluka) was added for 24 hours. Simvastatin was chemically activated before its use, as previously described. Endothelial cells were pretreated with actinomycin D (5 μg/mL) for 1 hour before treatment with Hcy for the mRNA stability assay.

Cardiovascular Microarray Analysis

After washing twice with PBS, cells were harvested by scraping. Atlas cardiovascular microarray (Clontech) was carried out according to the manufacturer’s instructions. Differentially displayed genes were studied in Hcy-treated HUVECs and control companion cells at the 3rd passage. The design of the cDNA array and the complete list of genes are available at www.clontech.com.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from HUVECs using Trizol reagent (Gibco BRL). Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out to amplify HMGCR gene in the presence of primers for an internal standard of 18S (Promega, primer pairs/competimers, 1:9), generating a product of 324 bp. cDNA was synthesized and amplified using Titan One-Step RT-PCR kit (Roche). HMGCR primers were 5’-TCCTTGGTGATGGAGCTTTG-3’ and 5’-TGCGAACCCTCTCATTTGCGAC-3’, generating a product of 290 bp (94°C for 30 sec, 60°C for 30 sec, and 68°C for 45 sec, 35 cycles). Products of RT-PCR were separated in 4% agarose gel and stained with ethidium bromide. Bands were quantified by computer-assisted densitometry. The ratios of intensity (after gel background subtraction) of the HMGCR gene bands versus that of the 18S bands were compared between the Hcy-treated and control conditions.

Western Blotting

HUVECs were lysed. HMGCR (polyclonal, kindly provided by Dr Peter Edwards, 1:1000), caveolin (polyclonal, Santa Cruz Biotech-nology, 1:2000), and tubulin (polyclonal, Sigma, 1:1000) proteins were detected as previously described.

Fluorescence Imaging

HUVECs were cultured on the glass coverslips and stained with filipin as previously described. After washing with PBS, anti-caveolin-1 antibody (10 μg/mL) was applied for 2 hours at room temperature, followed by Alexa Fluor 594-labeled secondary antibody (Molecular Probes) for 30 minutes. For quantitative analysis, HUVECs were seeded in 96-well plates (Falcon), treated, and stained as above. Fluorescence intensity was measured in a fluorescence plate reader. Data were normalized for cell density, as judged by the absorbance of crystal violet staining.

Total Intracellular Cholesterol Measurement

After incubation with or without 50 μmol/L Hcy for 24 hours, HUVECs were washed 3 times with PBS and lysed with 1% Triton X-100, and 10 μg of cholesterol-d7 (CDN Isotopes), an internal control, was added. Cellular proteins were determined with Pierce protein assay against BSA standards. Cellular and extracellular lipids were alkaline hydrolyzed and extracted with chloroform. Total cholesterol in the extracts was determined using Infinity Cholesterol Kit (Sigma) or gas chromatography mass spectrometry (GC-MS).

Determination of Cholesterol Level in Endothelial Cells and Media Using GC-MS

GC-MS was performed on a Hewlett-Packard 5970B mass selective detector using a Hewlett-Packard 5890A gas chromatograph. The GC column was a DB-5 MS (25 m, 0.2 mm ID, and 0.33-μm film thickness), and helium was the carrier gas. The oven temperature was set at 180°C for 1 minute, increased at 20°C/min to 250°C and then 5°C/min to 300°C, and held for 11.5 minutes. The injector, set at 270°C, was operated in splitless mode, and the detector transfer line was set at 280°C. The mass-selective detector was operated in electron ionization mode at 70 eV, and data were acquired using selected ion monitoring. The molecular ions of cholesterol and the cholesterol-d7 internal standard were monitored.

Measurement of NO Production with NO-Selective Microelectrodes

To determine NO production by the cultured cells, HUVECs were coincubated in 50 μmol/L Hcy with different concentrations of simvastatin for 24 hours. After a stable baseline current was recorded, cells were stimulated with A23187. The NO concentration was monitored with an optrode-electroplated, Nafion-coated, carbon fiber electrodes (30 μm outer diameter) using a highly sensitive potentiostat (InterMedical).

Statistical Analysis

All data are presented as mean±SEM of the number of replicative samples. ANOVA and t tests were used to determine any significant changes in densitometric values and NO production. Differences were considered significant at P<0.05.

Results

Homocysteine Increases the Expression of HMGCR in Endothelial Cells

We previously demonstrated that 24-hour exposure to 50 μmol/L Hcy resulted in endothelial dysfunction characterized by the reduced ability to generate NO in response to bradykinin or a calcium ionophore. This is the level (20 to 50 μmol/L) of Hcy found in patients with end-stage renal disease (ESRD). Therefore, HUVECs were cultured in the presence of 50 μmol/L Hcy for 24 hours before RNA isolation. Total RNA obtained from control and Hcy-treated cells was labeled, and hybridization reaction was performed...
on an array containing cardiovascular-relevant genes (600 genes). HMGCR message level was low in untreated cells but increased 1.6-fold in Hcy-treated cells (Figure 1A) above the confidence threshold of the technique (1.5-fold change). The increase in HMGCR mRNA was additionally confirmed by quantitative RT-PCR, which was normalized to 18S transcript (Figure 1B). The data showed that Hcy resulted in a 1.6- and 2.6-fold sustained increase in the abundance of HMGCR mRNA after 8- and 24-hour Hcy incubation, respectively. The posttranscriptional regulation of HMGCR mRNA was determined in the presence of the transcriptional inhibitor actinomycin D (5 μg/mL) (Figure 1C). Hcy (50 μmol/L) prolonged the half-life of HMGCR mRNA (τ1/2, 9.5±1.0 versus 5.0±0.2 hours).

The expression of HMGCR (97 kDa) and the time course of its induction by Hcy were examined by Western blot analysis at 4, 18, and 24 hours of continuous exposure to 50 μmol/L Hcy. HMGCR protein expression was 146±23.5% compared with control after 4 hours of exposure to Hcy and continued to rise to 271.9±31.3% and 277.2±33.4% after 18 and 24 hours, respectively. We have previously demonstrated that some of the effects of Hcy in HUVECs could be attenuated by coincubation with MnTBAP. Exposure of HUVECs to Hcy in combination with 50 μmol/L MnTBAP prevented the upregulation of HMGCR after 24 hours of coincubation. Simvastatin did not prevent the enzyme up-regulation (Figure 2A). Similar results were obtained in RMVECs (Figure 2B).

Effects of Hcy on Lipid Content of Endothelial Cells and Culture Media

The effects of Hcy on cholesterol production and secretion in HUVECs were determined. In HUVECs cultured with or without 50 μmol/L Hcy for 24 hours, intracellular cholesterol increased by 17.8±5.2%, as detected using GC-MS. Cholesterol content of the culture media was increased by 24±14.5% (Figure 3). Incubation with 0, 20, 50, and 100 μmol/L Hcy increased cellular total cholesterol in a dose-dependent manner by 22.2±7.3%, 39.5±1.2%, and 50.4±6.8% in HUVECs, respectively, as measured using the cholesterol oxidase method. Coincubation with 100 nmol/L simvastatin prevented cellular accumulation of cholesterol (Figure 4A). Similar findings were observed in RMVECs (Figure 4B).

Visualization of the cellular free cholesterol with filipin demonstrated its plasma membrane and perinuclear localiza-
accumulation of cholesterol was ruled out on the basis of the lack of colocalization of cholesterol with a lysosomal marker, LAMP2, or a mitochondrial tracker (data not shown). These data suggest that other compartments are involved in the cellular accumulation of cholesterol.

Several other regulators of intracellular cholesterol homeostasis (Figure 6) were also examined using cDNA microarray. The data demonstrated that Hcy resulted in a selective induction of HMGCR and caveolin-1 and was associated with endothelial accumulation of cholesterol.

Association of Free Cholesterol and Caveolin-1 in Hcy-Treated Endothelial Cells

We next examined whether the Hcy-induced expression of HMGCR and endothelial accumulation of cholesterol occurred in association with changes in caveolin-1 expression. As shown in Figure 2, HUVECs treated with 50 μmol/L Hcy for 4 to 24 hours showed an overexpression of caveolin-1 (2.3-fold increase). To examine cellular localization of cholesterol and caveolin-1, Hcy-treated HUVECs were costained with filipin and anti–caveolin-1 antibody. The data showed (Figure 7) that there was an increase in colocalization of filipin and caveolin-1 fluorescence in Hcy-treated cells compared with control. Of note, this colocalization was detectable not exclusively on the plasma membrane but occurred intracellularly.

HMGCR Inhibitor Prevents Hcy-Induced Inhibition of NO Production

As shown above, simvastatin reduced endothelial cholesterol synthesis in HHcy. We next examined the effect of this statin on agonist-evoked NO production in Hcy-treated endothelial cells. Preincubation with 50 μmol/L Hcy inhibited NO production by >51.4±2.7%. Coincubation with 10⁻⁸ and 10⁻⁶ mol/L simvastatin resulted in 72.4±4.8% and 81.7±5.0% recovery of A23187-stimulated NO release from Hcy-treated HUVECs, respectively (Figure 8).

Discussion

Screening HUVECs with cDNA microarray for the gene expression modified by Hcy revealed that HMGCR was upregulated. This finding was confirmed by quantitative RT-PCR of HMGCR message level and Western analysis of protein expression. The upregulation was in part attributable to increased HMGCR mRNA stability. Consistent with the increased expression of the enzyme, Hcy increased cholesterol production and caused its accumulation in endothelial cells despite a concomitant increase in cholesterol efflux to the culture medium. Endothelial accumulation of cholesterol may represent the missing link between elevated levels of plasma Hcy and development of atherosclerosis. Moreover, application of a cell-permeable superoxide dismutase mimetic reversed the Hcy-induced expression of HMGCR. HMGCR inhibitor, simvastatin, prevented intracellular accumulation of cholesterol and reversed the Hcy-induced suppression of NO production.

In general, cholesterol accumulation in the cell may be a result of an increased influx, de novo synthesis, or decreased efflux. Under our cell culture conditions, a possibility that
cholesterol accumulation reflects salvaging of extracellular cholesterol can be ruled out, because the culture medium used contained no cholesterol or cholesterol-containing lipoproteins. Similarly, cellular cholesterol retention attributable to a defect in the efflux pathway does not seem to be the case, because culture media conditioned by Hcy-treated HUVECs contained more cholesterol than control. This leaves the induction of HMGCR and increased cholesterol biosynthesis as the main cause of its cellular accumulation. Two aspects of Hcy-induced cholesterol accumulation in endothelial cells are of importance. First, the normal distribution of free cholesterol, lowest in the endoplasmic reticulum and highest in the plasma membrane, seems to be perturbed in Hcy-treated HUVECs. These cells show the predominant retention of cholesterol in numerous lipid droplets scattered around the cytosol. This compartment is distinct from endosomal-lysosomal or mitochondrial organelles. Second, it is surprising that HMGCR expression remained elevated for up to 24 hours despite the observed cellular and medium accumulation of cholesterol. The resulting cholesterol occupancy of the sterol-sensing domain in HMGCR should have accelerated its degradation (as seen in cholesterol-overloaded fibroblasts from patients with Niemann-Pick C disease).26 The fact that the net expression of the enzyme remains elevated and that this could be curtailed by a membrane-permeable SOD mimic argues that the induction of HMGCR is a persistent...
and a redox-dependent event in Hcy-treated HUVECs. This conclusion provides a rationale for the use of statins in HHCy.

The finding of caveolin-1 overexpression in Hcy-treated HUVECs seems to be related to the increased cholesterol biosynthesis. Caveolin or caveolin-enriched Triton-X insoluble membrane fractions have been implicated in the efflux of free cholesterol. Moreover, cytoplasmic free cholesterol is transported in particles containing palmitoylated caveolin to the cell surface. Recent observations link decreased caveolar cholesterol efflux, presumably via a decrease in caveolin expression, to cell-cycle progression. This is in line with our finding that an increased expression of caveolin-1 coincides with the cellular accumulation of cholesterol and can be viewed as an adaptive response, resulting in the observed augmentation of cholesterol efflux.

The leading mechanism suggested for the adverse vascular effects of homocysteine on endothelial function involves oxidant stress resulting in a reduced bioavailability of NO. Homocysteine, like other thiol-containing amino acids, undergoes auto-oxidation with the generation of ROS. ROS, such as superoxide anion, interact with NO to form peroxynitrite, leading to a depletion of biologically active NO. Our data show that the superoxide dismutase mimetic MnTBAP reversed the upregulation of HMGCR expression. These finding underscore the importance of a superimposed oxidant stress in Hcy-induced cholesterol dysregulation.

Statins decrease plasma cholesterol and are known to reduce morbidity and mortality from coronary artery disease even in patients with average cholesterol levels. A growing number of studies indicate that clinical benefits of statins are related to pleiotropic effects on endothelial function, inflammation, coagulation, and plaque vulnerability. Recently, Llevadot et al and Dimmeler et al demonstrated that statins could mobilize and differentiate endothelial cell precursors after ischemic injury. These clinical benefits are apparently unrelated to the hepatic effect of statins in reducing serum lipid profiles. In most of these studies, the effect of statins has been ascribed to the inhibition of the mevalonate-dependent geranylgeranylation of Rho GTPase proteins and activation of Akt signaling. Although HHCy is not associated with abnormalities in serum lipid levels, our present study provides biochemical evidence of impairment of cholesterol metabolism in endothelial cells and highlights the long-sought link between HHCy and progression of atherosclerosis. These data provide a rationale for the potential benefits of statins in patients with HHCy, even without hyperlipidemia.

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