Homocysteine Enhances Endothelial Apoptosis via Upregulation of Fas-Mediated Pathways

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Abstract—Hyperhomocysteinemia is an independent risk factor for the development of atherosclerosis. However, the underlying mechanism of endothelial cell injury in hyperhomocysteinemia has not been elucidated. In this study, we examined the effect of homocysteine (Hcy) on Fas-mediated apoptosis in endothelial cells. Hcy-induced upregulation of Fas in endothelial cells (ECs) in a dose-dependent manner. At the same time, Hcy increased intracellular peroxide in ECs. Hcy-induced Fas expression was inhibited by the treatment with catalase. Hcy increased NF-κB DNA binding activity, and adenovirus-mediated transfection of a Iκ-B mutant (Iκ-B mt) gene inhibited Hcy-induced Fas expression. ECs were sensitive to Fas-mediated apoptosis when exposed to Hcy. Under these conditions, Iκ-B mt protected ECs from Fas-mediated apoptosis. In addition, Hcy inhibited expression of the caspase-8 inhibitor FLICE-inhibitory protein (FLIP). Adenovirus-mediated transfection of constitutively active Akt gene abolished the Hcy-mediated downregulation of FLIP. These data suggest that upregulation of Fas expression and downregulation of FLIP is a mechanism through which Hcy induces EC apoptosis. (Hypertension. 2004;43:1208-1213.)

Key Words: endothelium ■ apoptosis ■ oxidative stress ■ protein kinases

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erhomocysteinemia is an independent risk factor for atherosclerosis and vascular disease. Elevated plasma homocysteine (Hcy) level is associated with 3-fold increase in the risk of acute myocardial infarction. Hcy is an amino acid derived from the metabolic demethylation of dietary methionine. Hyperhomocysteinemia is caused by homozygous deficiency of the gene encoding cystathionine b-synthetase or methionine. Hyperhomocysteinemia is an independent risk factor for the development of atherosclerosis. However, the underlying mechanism of endothelial cell injury in hyperhomocysteinemia has not been elucidated. In this study, we examined the effect of homocysteine (Hcy) on Fas-mediated apoptosis in endothelial cells. Hcy-induced upregulation of Fas in endothelial cells (ECs) in a dose-dependent manner. At the same time, Hcy increased intracellular peroxide in ECs. Hcy-induced Fas expression was inhibited by the treatment with catalase. Hcy increased NF-κB DNA binding activity, and adenovirus-mediated transfection of a Iκ-B mutant (Iκ-B mt) gene inhibited Hcy-induced Fas expression. ECs were sensitive to Fas-mediated apoptosis when exposed to Hcy. Under these conditions, Iκ-B mt protected ECs from Fas-mediated apoptosis. In addition, Hcy inhibited expression of the caspase-8 inhibitor FLICE-inhibitory protein (FLIP). These data suggest that upregulation of Fas expression and downregulation of FLIP is a mechanism through which Hcy induces EC apoptosis.

In this study, we examined the pathological role of Hcy in Fas-mediated apoptosis in ECs. We found that Hcy-induced apoptosis resulted from the upregulation of Fas and the downregulation of FLICE-inhibitory protein (FLIP). These data suggest that Hcy is toxic to ECs by sensitizing these cells to the Fas death pathway.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were isolated as described. HUVECs were maintained in endothelial growth medium (EGM-2; Clonetics, San Diego, Calif) containing 2% fetal bovine serum (FBS) on collagen-coated dishes (IWAKI, Japan).

Adenoviral Constructs

Replication-defective adenovirus vectors expressing constitutively active form of murine Akt (Adeno-myrAkt) from the cytomegalovirus (CMV) promoter were described previously. Adenovirus vector expressing mutant Iκ-B (S32A/S36/A) gene from the CMV promoter was a kind gift from Dr Michio Tamatani (Osaka University, Japan). All viral constructs were grown in 293 cells and purified by CsCl gradient ultracentrifugation. Viral titers were determined by plaque assay.

Protein Detection by Flow Cytometry

To analyze cell-surface expression of Fas, harvested cells were incubated with 10 μg/mL mouse antibody against Fas (UB2, MBL)
or mouse IgG for 1 hour at 4°C. Next, cells were incubated with 10 μg/mL FITC-conjugated rat anti-mouse IgG (Pharmingen) for 30 minutes at 4°C. Immunofluorescence staining on the cell surface was analyzed by flow cytometry on the FL-1 channel.

**Analysis of Fas mRNA**

Total RNA from ECs was extracted by a guanidine isothiocyanate/acid phenol method. Poly(A)⁺ RNA was prepared using Oligo(dT)-Latex (Takara Biomedicals, Japan). Northern blot analysis was performed as previously described. The probe DNA of Fas a 2.5-kb XhoI fragment contained human Fas cDNA. The cDNA probe for human Fas and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) were labeled with [³²P]dCTP (111 TBq/mmol) by using the multiprime DNA labeling kit (Amersham).

**Electrophoretic Mobility Shift Assay of NF-κB**

Nuclear proteins were prepared from HUVECs, as described. Oligonucleotides containing NF-κB consensus binding site (5'-GGG GAC TTT CCC-3') were labeled using polynucleotide kinase and [γ-³²P] ATP; 10 μg nuclear extracts were incubated with 1 μL labeled DNA probe for 20 minutes on ice. Samples were separated in 5% acrylamide gel.

**Western Immunoblot Analysis**

Protein extract (20 μg) was fractionated on SDS-polyacrylamide electrophoresis gel and transferred to a polyvinylidine difluoride membrane (Immobilon-P, Millipore). The membrane incubated with primary antibody (anti-phospho-Akt [Cell Signaling], anti-Akt1 [Santa Cruz], anti-α-tubulin [Carbiochem]) overnight at 4°C. Mouse monoclonal antibody against human FLIP (NF6) was a gift from Dr Marcus E. Peter (The Ben May Institute for Cancer Research, University of Chicago, Ill). Then, the membrane was incubated with secondary antibody (anti-mouse, anti-rabbit, or anti-goat IgG horse-radish peroxide conjugate [Promega]) for 1 hour. The immune complexes were detected by chemiluminescence methods (ECL, Amersham).

**Measurement of Intracellular Peroxide**

After various treatments, cells were incubated with 10 μL/10-cm² dish of 2', 7'-dichlorofluorescin diacetate for 30 minutes in CO₂ incubator. Harvested cells were suspended in 500 μL PBS. Intracellular peroxide was analyzed by flow cytometry on FL-1 channel.

**Cell Viability Assays**

Attached and floating ECs were fixed in cold 90% ethanol for 20 minutes and then resuspended in staining buffer consisting of 1 mg/mL RNaseA, 20 μg/mL propidium iodide, and 0.01% NP40. DNA content was analyzed by flow cytometry on FL-2 channel and gating was set to exclude debris and cellular aggregates.

**Statistical Analysis**

Statistical analysis was performed by 1-way ANOVA. Results are expressed as mean±SEM. A value of P<0.05 was considered significant.

**Results**

**Homocysteine Induces Upregulation of Fas Expression Through Enhancement of Peroxide Production in ECs**

To define the molecular mechanism underlying endothelial injury by homocysteine, we first examined whether homocysteine modulates the levels of Fas, a death receptor, in ECs. As shown in Figure 1A, homocysteine induced a dose-dependent increase in the levels of cell surface Fas expression in ECs. Homocysteine also induced an upregulation of Fas mRNA expression. A maximum increased level of Fas mRNA expression was seen at 12 hours after stimulation with homocysteine (Figure 1B). However, coincubation with catalase, a scavenger of peroxide, inhibited this upregulation. Additionally, homocysteine induced a dose-dependent increase in the levels of intracellular peroxide, and this increase was suppressed by coincubation with catalase (Figure 1C). These results suggest that peroxide is involved in the mechanism by which homocysteine upregulates Fas expression.

**Homocysteine Induces Upregulation of Fas Expression Through Activation of a Transcriptional Factor NF-κB**

We next examined whether activation of NF-κB involves homocysteine-induced upregulation of Fas expression by using adenoviral vector encoding mutant IκB (mt IκB) that contains serine-to-alanine mutations at amino acids 32 to 36.
(IκBα S32A/S36A) and inhibits phosphorylation and proteasome-mediated degradation of IκB. To demonstrate that expression of mt IκB effectively inhibits NF-κB binding activity in ECs, we performed electrophoretic mobility shift assays with nuclear extracts and a consensus NF-κB DNA binding site. As shown in Figure 2, infection of adenoviral vector encoding mt IκB suppressed NF-κB DNA binding activity in ECs treated with homocysteine or H₂O₂, whereas adenoviral vector expressing LacZ had no effect. Additionally, transfection of mt IκB inhibited upregulation of Fas protein and mRNA expression induced by homocysteine, whereas control vector had no effect (Figure 3A and 3B).

Homocysteine Sensitizes ECs to Fas-Mediated Apoptosis via NF-κB–Dependent Pathways

We next examined whether homocysteine-induced upregulation of Fas expression results in an increment of EC apoptosis. Incubation with an agonistic antibody CH11 significantly increased the levels of apoptosis in ECs pretreated with homocysteine (Figure 4). CH11, however, did not increase the levels of apoptosis without homocysteine pretreatment (data not shown). Consistent with the results in Figure 3A and B, transfection of mt IκB significantly inhibited the induction of apoptosis by CH11 in homocysteine-pretreated cells, whereas control vector had no effect (Figure 4). These results suggest that homocysteine sensitizes Fas-mediated apoptosis through activation of NFκB-mediated pathways.

Homocysteine Downregulates Cellular FLIP Expression and Sensitizes Fas-Mediated Apoptosis Through Inhibition of Akt Activity

NF-κB activation has been shown to upregulate cellular FLIP (cFLIP), an endogenous caspase 8 inhibitor, resulting in increased resistance to Fas-mediated apoptosis.²⁵,²⁶ Thus, we examined whether homocysteine upregulates cFLIP expres-
Although homocysteine induces NF-κB activation as shown in Figure 2, it did not upregulate, but downregulated cFLIP expression in ECs (Figure 5A). Because we previously demonstrated that cFLIP expression is regulated by Akt-mediated pathways,46,47 we next examined whether homocysteine downregulates through modulation of Akt activity. As shown in Figure 5B, homocysteine induced suppression of Akt activity in ECs. Importantly, activation of Akt exogenously by injection of adenoviral construct expressing the constitutively active Akt (myrAkt) reversed the downregulation of cFLIP expression by homocysteine. Additionally, VEGF, an activator of endogenous Akt, also reversed this downregulation. Furthermore, infection of Ad-myrAkt reversed agonistic antibody against (CH11)-induced apoptosis in ECs preincubated with homocysteine, whereas control vector had no effect (Figure 5C).

These findings suggest that homocysteine sensitizes Fas-mediated apoptosis through suppression of Akt-mediated pathways as well.

**Homocysteine Promotes EC Apoptosis via Fas/Fas Ligand-Mediated Pathway in Autocrine/Paracrine Manner**

As shown in Figure 5C, Hcy directly induced EC apoptosis in the absence of an agonistic antibody against Fas (CH11). To examine the participation of Fas-mediated death pathway in Hcy-induced EC apoptosis, ECs were incubated with Hcy in the presence or absence of a neutralizing antibody against Fas (ZB4). The preincubation and coincubation of ZB4 partially inhibited Hcy-induced EC apoptosis dose-dependently (Figure 6). The treatment of control IgG had no effect on Hcy-induced EC apoptosis. This result shows that Hcy-induced EC apoptosis occurs via Fas-mediated death signaling in autocrine/paracrine manner.

**Discussion**

In the present study, we demonstrated the following findings on the mechanism responsible for the endothelial damage by homocysteine. Homocysteine upregulates the expression of Fas, a death receptor, in ECs via NF-κB activation. Downregulation of NF-κB activity by expressing mutant IkBα prevents homocysteine-induced sensitization to Fas-mediated apoptosis in ECs. Homocysteine suppresses the expression of cFLIP, an endogenous inhibitor of the caspase 8, through the downregulation of Akt, a survival signal for EC. Upregulation of Akt by expressing the constitutively active form of Akt prevents homocysteine-induced EC apoptosis through the restoration of cFLIP expression.

Hyperhomocysteinemia has been recognized as an independent risk factor that predicts adverse cardiovascular
may be one of the pathological mechanisms in homocysteine-induced EC injury and that Akt can be a potential therapeutic target in hyperhomocysteinemia.

EC apoptosis represents the critical event for the initiation of atherosclerosis. The concept of an association between EC apoptosis and the development of atherosclerosis has been supported by the hypothesis that disturbed shear-stress on vessel wall or adhesive platelets participate in EC apoptosis. Recent direct evidence demonstrated that EC apoptosis increases in the downstream part of atherosclerotic plaque whereas blood flow-mediated shear stress is low. Also, shed membrane particles including apoptotic ECs are elevated in plasma of patients with unstable angina and acute myocardial infarction. These findings show that EC apoptosis is involved in the determination of the susceptibility to atherosclerotic lesion development. Thus, our observation in this study suggests a new mechanistic link between EC apoptosis and the susceptibility of atherosclerotic plaque in patients with hyperhomocysteinemia.

In conclusion, these observations suggest a new mechanism by which homocysteine may exert its proatherogenic effects in patients with cardiovascular diseases.

**Perspectives**

Homocysteine induces endothelial apoptosis via upregulation of Fas and downregulation of FLIP, an endogenous inhibitor of caspase-8, which might provide new insight into the pathological mechanism in hyperhomocysteinemia.

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**References**


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