Nitric Oxide Inhibition of Homocysteine-induced Human Endothelial Cell Apoptosis by Down-regulation of p53-dependent Noxa Expression through the Formation of S-Nitrosohomocysteine*

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Hyperhomocysteinemia is believed to induce endothelial dysfunction and promote atherosclerosis; however, the pathogenic mechanism has not been clearly elucidated. In this study, we examined the molecular mechanism by which homocysteine (HCy) causes endothelial cell apoptosis and by which nitric oxide (NO) affects HCY-induced apoptosis. Our data demonstrated that HCY caused caspase-dependent apoptosis in cultured human umbilical vein endothelial cells, as determined by cell viability, nuclear condensation, and caspase-3 activation and activity. These apoptotic characteristics were correlated with reactive oxygen species (ROS) production, lipid peroxidation, p53 and Noxa expression, and mitochondrial cytochrome c release following HCY treatment. HCY also induced p53 and Noxa expression and apoptosis in endothelial cells from wild type mice but not in the p53-deficient cells. The NO donor S-nitroso-N-acetylpenicillamine, adenoviral transfer of inducible NO synthase gene, and antioxidants (α-tocopherol and superoxide dismutase plus catalase) but not oxidized SNAP, 8-Br-cGMP, nitrite, and nitrate, suppressed ROS production, p53-dependent Noxa expression, and apoptosis induced by HCY. The cytotoxic effect of HCY was decreased by small interfering RNA-mediated suppression of Noxa expression, indicating that Noxa up-regulation plays an important role in HCY-induced endothelial cell apoptosis. Overexpression of inducible NO synthase increased the formation of S-nitroso-HCY, which was inhibited by the NO synthase inhibitor N-nitroso-L-arginine. Moreover, S-nitroso-HCY did not increase ROS generation, p53-dependent Noxa expression, and apoptosis. These results suggest that up-regulation of p53-dependent Noxa expression may play an important role in the pathogenesis of atherosclerosis induced by HCY and that an increase in vascular NO production may prevent HCY-induced endothelial dysfunction by S-nitrosylation.

Homocysteine (HCy)1 is a thiol-containing amino acid that is formed when methionine is converted to cysteine. Once synthesized, HCY may either be metabolized to cysteine by the transulfuration pathway or remethylated to methionine (1). Deficiency of the enzyme involved in these pathways (i.e. cystathionine β-synthase or 5,10-methylenetetrahydrofolate reductase) and/or a cofactor (i.e. folate, vitamin B12, or vitamin B6) required for HCY metabolism leads to elevations in the plasma concentration of HCY and its precursor methionine as well as a reduction in plasma concentrations of cysteine. Hyperhomocysteinemia is a significant cardiovascular risk factor, the strength of which is almost equal to that of hyperlipidemia (2).

Recent studies of cultured cells in vitro have showed that HCY causes endothelial dysfunction such as apoptotic cell death (3), impairment of platelet aggregation (4), expression of procoagulant molecules (5), oxidation of low density lipoprotein (6), and inhibition of thrombogenic protein kinase C activation (7). These adverse vascular effects of HCY are caused by the generation of reactive oxygen species (ROS) as a consequence of autodisulfidation of its sulfhydryl group (6) and/or a decrease in endothelial nitric oxide (NO) bioavailability via the formation of S-nitroso-HCY (8). Furthermore, earlier studies have demonstrated that HCY causes abnormal endothelium-dependent vasorelaxation and endothelial cell injury when administered to mice (9), rats (10), and non-human primates (11). This suggests that HCY-induced endothelial dysfunction may predispose to atherosclerotic and vascular disease by altering the normally antithrombotic and vasoprotective phenotype of vascular endothelium (12).

NO produced by endothelial NOS has multiple beneficial effects including modulation of platelet aggregation, inhibition of leukocyte adhesion, and control of vascular smooth muscle cell proliferation (13). It is now clear that NO can induce apoptosis in some cells or prevent apoptosis in others. Our previous study has shown that NO produced from NOS or chemical drugs can rescue cells from apoptosis by inactivating caspase-3 via S-nitrosylation (14). Therefore, a de-

1 The abbreviations used are: Hcy, homocysteine; HUVEC, human umbilical vein endothelial cell; NO, nitric oxide; NOS, nitric oxide synthase; SNAP, S-nitroso-N-acetyl-L-penicillamine; OxSNAP, oxidized SNAP; Z-VAD-fmk, benzoxylcarbonyl-Val-Ala-Asp-fluoromethyl ketone; NMA, 1-N-nitroso-L-arginine; ROS, reactive oxygen species; SOD, superoxide dismutase; pNA, p-nitroanilide; Ac, N-acetyl; AdiNOS, adenoviral iNOS; DCF-DA, dichlorofluorescin diacetate; S-NHOCy, S-nitrosohomocysteine; MDA, malondialdehyde; PBS, fetal bovine serum; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcription; PBS, phosphate-buffered saline; iNOS, inducible NOS; cho, aldehyde.
crease in the bioavailability of NO is associated with an increase in the vascular inflammation and the proliferation of smooth muscle cells, all of which have been shown to play an important role in the development of atherosclerosis. These evidences indicate that NO is a key molecule in preventing endothelial dysfunction and atherosclerosis as well as promoting vascular relaxation.

Extensive studies have indicated that HCy can cause endothelial dysfunction and vascular lesion formation; however, the cellular and molecular mechanisms between hyperhomocysteinemia and vascular disease are poorly understood. In this study, we investigated the molecular mechanism by which HCy causes endothelial cell apoptosis and the protective function of NO against HCy-induced endothelial cell death. We here found that HCy induced endothelial cell apoptosis by cytochrome c release and caspase-3 activation via up-regulation of p53 and Noxa. Furthermore, increased NO production by NO donor or iNOS gene transfer ameliorated the endothelial cell injury induced by HCy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dichlorofluorescin diacetate (DCF-DA) was purchased from Molecular Probes, Inc. (Eugene, OR). Dulbecco’s modified Eagle’s medium, M199, penicillin, streptomycin, t-glutamate, and heparin were obtained from Invitrogen. Basic fibroblast growth factor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). N-acetyl-Tyr-Val-Ala-Asp-p-nitroanilide (Ac-DEVD-pNA) and iNOS substrate (Ac-DEVD-pNA) were obtained from Alexis Corp. (San Diego, CA). Antibodies against p53, cytochrome c, iNOS, and actin were purchased from Transduction Laboratories (Lexington, KY). Antibodies against caspase-8 and caspase-3 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). S-nitroso-N-acetyl-dl-penicillamine (SNAP) and S-nitrosohemocystine (S-NOHc) were synthesized, as described previously (6, 15). All other reagents were purchased from Sigma-Aldrich and used as recommended by the manufacturer.

**Cell Culture**—HUVECs were isolated as described previously (16). The cells were grown on a gelatin-coated 75-cm² flask in M199 with 20% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 3 ng/ml basic fibroblast growth factor, and 5 units/ml heparin at 37 °C under 5% CO₂, 95% air. The cells used in this study were between passages 3 and 7. Mouse endothelial cells were isolated from lungs of wild type and p53-deficient mice. In brief, animals were killed by cervical dislocation, and lungs were collected in ice-cold Dulbecco’s modified Eagle’s medium. Peripheral lung tissue was minced and digested by 100 µg/ml collagenase-A (Roche Applied Science). Harvested cells were washed with PBS and resuspended in murine lung endothelial cell medium (37 °C) containing 20% fetal bovine serum until cell density was reached to 5 × 10⁵ cells/well. Cells used in the experiments were plated in 0.1% gelatin-coated T75 flasks. Cells were washed after 24 h and cultured for 2–4 days. Magnetic beads were coated with anti-mouse CD102 (clone 3C4; Pharmingen) antibody (5 µg/4 × 10⁶ beads; Dynabeads M-450; Dynal). Per flask, 4 × 10⁶ beads were added and incubated for 1 h at 4 °C. Cells were trypsinized and selected in a magnetic field for 10 min. Cultures were grown to confluence and selected twice before being plated for experiments. Cells used in the experiments were on average, 10 days in culture.

**Cell Viability Assay**—Cell viability was determined by the crystal violet staining method, as described previously (14). In brief, HUVECs (2 × 10⁵ cells/well) were plated onto 6-well plates in 2 ml of M199 containing 20% fetal bovine serum until cell density was reached to 70–80% confluencc. Cells were cultured with serum-free medium for 6 h and switched to 5% fetal bovine serum M199 medium containing HCy. After 16 h, viable cells were stained by a crystal violet staining method. Plates were washed four times with tap water. After drying, cells were lysed with 1% SDS solution and dye was extracted at 550 nm using a 96-well plate reader. Cell viability was calculated from relative dye intensity compared with untreated samples.

**Detection of Cytochrome c Release**—Harvested cells were resuspended in ice-cold mitochondrial fractionation buffer (20 mM HEPES, 1 mM sodium EDTA, 10 mM KCl, 1 mM dithiothreitol, 10 µM aprotinin, 10 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose). After homogenization using a Dounce homogenizer, cytosolic fractions were obtained by centrifugation at 12,000 × g for 20 min at 4 °C (14). The cytosolic proteins (40 µg) were loaded onto a 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting was performed using a cytochrome c antibody to determine mitochondrial cytochrome c release.

**Caspase Activity Assay**—Cells were harvested with ice-cold PBS, and resuspended in 100 mM HEPES buffer (pH 7.4) containing an inhibitor mixture (5 mg/ml aprotinin and peptatin, 10 mg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). The cell suspension was lysed by three freeze-thaw cycles, and the cytosolic fraction was obtained by centrifugation at 12,000 × g for 10 min at 4 °C. Caspase activities were determined by measuring proteolytic cleavage of chromogenic substrate Ac-DEVD-pNA (caspase-3-like activity), Ac-IETD-pNA (caspase-8-like activity), or Ac-LEHD-pNA as described previously (14).

**Western Blot Analysis**—Cells resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture were lysed by three cycles of freeze and thaw, and the cytosolic fractions were obtained by centrifugation at 100,000 × g for 40 min. Cytosolic proteins (40 µg) were separated on a SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against p53, caspase-3, caspase-8, p53, cytochrome c, and iNOS, and the protein bands were visualized by exposure to x-ray film, as described previously (15).

**Reverse Transcription (RT)-PCR Analysis**—Total RNAs from HUVECs and their cDNA were prepared by previous methods (17). Three µl of the cDNA mixture was used for PCR in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 µM of each of the primers for iNOS and Noxa. The PCR amplification was performed in a DNA thermal cycler under the following conditions: denaturation at 94 °C for 5 min for the first cycle and for 30 s starting from the second cycle, annealing of p53 at 62 °C for 30 s, annealing of Noxa at 52 °C for 30 s, and extension at 72 °C for 30 s for 30 cycles. Final extension was performed at 72 °C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The primers used were 5′-GACGCCATGGTTGACGTTGAACTCTGCTGTA-3′ (sense) and 5′-CTATGTCAGAAATGTTCGTCGTCATT-3′ (antisense) for the p53, 5′-CCGGTGATCATCTTCTGCTCCAGAGAG-3′ (sense) and 5′-CCGGATTCTCAGTTCTGCTGCAGCAGAG-3′ (antisense) for the Noxa, and the primer pairs were 5′-CAGCACCGTGGAAGCC-3′ (sense) and 5′-CAGCAGTGAGAAGCCGGGAGG-3′ (antisense) for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

**Determination of ROS and Lipid Peroxidation**—ROS was determined byDCF fluorescence by use of DCF-DA. HUVECs were treated with HCy for 30 min and incubated with 10 µM H₂DCF-DA for another 30 min. Cells were washed with PBS twice, and fluorescence was analyzed using a confocal microscope. Lipid peroxidation was determined by measuring thiobarbituric acid-reacting substances using malondialdehyde (MDA) standards as described previously (18).

**Transfection with siRNA**—The target sequence of siRNAs for Noxa was 5′-AACCTCCGGGCGAACTCTGCTG-3′. Endothelial cells were transfected with these double-stranded siRNAs (40 nM/dl) for 12 h by the Lipofectamine method according to the manufacturer’s protocol (Invitrogen) and recovered in M199 medium containing 20% fetal bovine serum for 30 h. Cells were cultured with serum-free medium for 6 h, followed by treatment with 1 mM HCy in M199 medium containing 5% fetal bovine serum. After 16 h, viable cells were stained by a crystal violet staining method.

**Determination of NO and S-NOHCy**—The level of nitrite, a stable product of NO, was determined in cell culture medium by Griess reagents (14). The level of S-NOHc was measured by Saville’s method (19). Cells for S-NOHc measurements were incubated with 1 mM HCy in M199 medium containing 5% fetal bovine serum. After 16 h, viable cells were stained by a crystal violet staining method.

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**4′,6-Diamidino-2-phenylindole (DAPI) Staining of Nuclei**—Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome, DAPI. HUVECs were grown on glass coverslips and treated with HCy in the presence or absence of SNAP and Z-VAD-fmk for 12 h. Cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde for 30 min. Following washing with PBS, cells were incubated in a DAPI solution (1 µg/ml) for 30 min in the dark. Cells were then washed with PBS and subjected to fluorescence microscopy.

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Fig. 1. Hcy induces caspase-dependent HUVEC death. HUVECs were plated on a 12-well plate at a density of $2 \times 10^5$ cells/well in 2 ml of M199 containing 20% FBS and cultured until cell density reached 70–80% confluence. Cells were cultured with serum-free medium for 6 h. A, cells were incubated with various concentrations of Hcy in 5% FBS-containing culture medium. After 16 h, cell viability was measured by a crystal violet staining method. B, cells were treated with 1 mM Hcy in the presence or absence of Z-VAD-fmk, Ac-IETD-cho, or Ac-DEVD-cho for 16 h. Cell viability was measured by a crystal violet staining method. C, HUVECs were grown on glass coverslips and treated with 1 mM Hcy in the presence or absence of Z-VAD-fmk (100 $\mu$M) or Ac-DEVD-cho (100 $\mu$M) for 12 h. Changes of nuclear morphology were assessed by DAPI staining. Data shown in A and B are the mean ± S.D. ($n = 4$), * $p < 0.05$; ** $p < 0.01$ versus untreated control (A) and Hcy alone (B).

Results

HCy Induces Caspase-3-dependent HUVEC Apoptosis—To examine the effect of Hcy on endothelial cell survival, HUVECs were cultured with different concentrations of Hcy, and cell viability was measured by a crystal violet staining method. Hcy decreased HUVEC viability in a dose-dependent manner and appeared to exert maximal effect at a concentration of 1 mM (Fig. 1A). This effect was decreased by the addition of the broad spectrum caspase inhibitor Z-VAD-fmk, the caspase-9 inhibitor Ac-LEHD-cho, and the caspase-3 inhibitor Ac-DEVD-cho, but not by the caspase-8 inhibitor Ac-IETD-cho (Fig. 1B), indicating that activation of caspase-9 and -3 is involved in Hcy-induced endothelial cell death. Furthermore, HUVECs undergoing Hcy-mediated cell death displayed typical apoptotic features, including chromatin condensation, nuclear pyknosis, and in some cases nuclear fragmentation compared with control (Fig. 1C, arrow). These morphological changes were decreased by co-treatment with Z-VAD-fmk and Ac-DEVD-cho, demonstrating that Hcy caused caspase-dependent apoptotic cell death in cultured HUVECs.

SNAP and Antioxidant Suppress Hcy-induced Apoptosis—Endothelial cells produce NO, which has been shown to act as an endogenous apoptotic inhibitor (20), antioxidant (21), and vasodilator (13). To examine the roles of NO and antioxidants in endothelial cell apoptosis induced by Hcy, HUVECs were treated with 1 mM Hcy in the presence or absence of the chemical NO donor SNAP, the NOS inhibitor NMA, and antioxidants α-tocopherol and SOD plus catalase, and cell viability was measured. SNAP treatment prevented endothelial cell death caused by Hcy, in a dose-dependent manner (Fig. 2, A and B). Furthermore, NMA slightly increased apoptotic cell death, and the ROS-scavenging systems, such as α-tocopherol and SOD plus catalase, suppressed Hcy-induced apoptosis (Fig. 2, A and B). It has been demonstrated that NO produces cGMP from GTP by activating heme-containing soluble guanylate cyclase and is oxidized to nitrite and nitrate (22). We next examined the effect of oxidized SNAP (OxiSNAP, which has exhausted all of the NO and does not liberate NO), nitrite, nitrate, and 8-Br-cGMP on Hcy-induced HUVEC apoptosis. Treatments with these chemicals did not show any protective effect compared with that of SNAP (Fig. 2C).

NO Produced by iNOS Gene Transfer Inhibits Hcy-induced HUVEC Apoptosis—We have previously shown that iNOS gene transfer prevented endothelial cell apoptosis induced by lipopolysaccharide (20). Since the NOS inhibitor NMA slightly increased Hcy-induced endothelial cell apoptosis (Fig. 2), we further examine whether the increased endogenous NO production protects endothelial cells from Hcy-induced apoptosis. HUVECs were treated with Hcy following transfection with AdiNOS, and cell viability was measured. AdiNOS-transfected cells expressed iNOS protein and significantly increased NO production compared with control (Fig. 3A). When the transfected cells were treated with the specific NOS inhibitor NMA, NO production was significantly inhibited without changing the iNOS protein level. The transfected cells significantly prevented HUVECs from Hcy-induced apoptotic cell death compared with control, and this prevention was reversed by the addition of NMA (Fig. 3B).

HCy Increases Intracellular ROS Generation and Lipid Peroxidation, Which Are Inhibited by NO and Antioxidants—HCy
undergoes autoxidation and leads to the formation of ROS, which potentiates oxidative lipid peroxidation and cell injury (6), resulting in caspase-dependent apoptosis (23). We investigated the effect of NO and antioxidants on HCy-mediated intracellular ROS accumulation by measuring the amount of fluorescent DCF produced from the oxidative reaction of DCF-DA with H$_2$O$_2$. HUVECs treated with HCy resulted in a significant increase in intracellular ROS accumulation compared with control cells, and this increase was further enhanced in the presence of NMA and reduced by co-treatment with SNAP and SOD plus catalase (Fig. 4, A and B). Furthermore, AdiNOS transfection significantly reduced the HCy-mediated increase in intracellular ROS accumulation, which was reversed by the addition of NMA (Fig. 4, A and C). Since ROS is responsible for the production of MDA, as an index of oxidative damage, via oxidative lipid peroxidation (6), we examined the effects of NO and antioxidants on HCy-mediated MDA production. Treatment of HUVECs with HCy increased the production of MDA, which was inhibited by treatment with SNAP...
and SOD plus catalase but partially augmented by NMA (Fig. 4D). Furthermore, AdiNOS-transfected cells suppressed HCy-induced lipid peroxidation, and this suppression was reduced by the addition of NMA. These results indicate that NO is able to suppress HCy-induced ROS generation and oxidative injury in cultured HUVECs.

HCy Induces p53 and Noxa Expression and Apoptotic Signal Cascade, Which Are Suppressed by NO and Antioxidants—Several lines of evidence showed that oxidative stress is highly correlated with the expression levels of p53 and the BH3-only protein Noxa in a number of situations (24, 25), resulting in mitochondrial cytochrome c release and activation of caspase-dependent apoptotic signaling pathways in cultured endothelial cells. We first examined whether HCy would regulate p53 and Noxa expression in HUVECs. Incubation of HUVECs with HCy resulted in up-regulation of p53 protein and mRNA levels as well as Noxa expression in a dose-dependent manner (Fig. 5A). We also examined whether NO and antioxidants would regulate the effects of HCy on the expression of p53 and Noxa. Treatment of HUVECs with SNAP, α-tocopherol, and SOD plus catalase, but not with OxiSNAP, blocked HCy-induced increases in the mRNA and protein levels of p53 and Noxa (Fig. 5B) but not the Bcl-2 family proteins Bcl-2 and Bax (data not shown). We further examined whether HCy would regulate mitochondrial cytochrome c release and caspase activation and whether this regulation could be modulated by NO and antioxidants. Western blot analyses showed that HCy treatment increased the redistribution of mitochondrial cytochrome c to the cytosol and caspase-3 activation but not caspase-8 activation (Fig. 5C). These increases were blocked by co-treatment with SNAP, α-tocopherol, and SOD plus catalase but not by OxiSNAP. Furthermore, HCy treatment increased caspase-9-like (LEHDase) and caspase-3-like (DEVDase) activities but did not exert a significant increase in caspase-8-like (IETDase) activity (Fig. 5D). These results suggest that HCy requires cytochrome c release and caspase-3 activation, but not caspase-8 activation, for inducing HUVEC apoptosis via the up-regulation of p53-dependent Noxa expression.

Endothelial Cells from p53−/− Are Resistant to HCy-induced Apoptosis—To obtain direct evidence for the involvement of ROS-mediated p53 and Noxa expression in HCy-induced apoptosis, we examined the effect of HCy on their expression and apoptosis of endothelial cells from p53−/− and p53+/− mice. Incubation of p53−/− endothelial cells with HCy exhibited significant increases in both p53 and Noxa expression, which were inhibited by AdiNOS transfection, and the effect of AdiNOS was reversed by the addition of NMA (Fig. 6A). However, p53 and Noxa were not detectable in p53−/− endothelial cells when
the cells were incubated with HCY regardless of iNOS expression. Under the same experimental conditions, HCY increased apoptosis of p53<sup>−/−</sup> endothelial cells by about 40% compared with minimal apoptosis (less than 15%) of p53<sup>+/+</sup> cells (Fig. 6B). The apoptotic response of p53<sup>−/−</sup> endothelial cells to HCY was significantly inhibited by AdiNOS transfection, whereas iNOS gene transfer did not nearly affect cell viability of p53<sup>−/−</sup> endothelial cells. The inhibitory effect of AdiNOS was reversed by the addition of NMA.

siRNAs for Noxa Suppressed HCY-induced Cell Death—To test whether up-regulation of Noxa plays an important role in HCY-induced apoptosis, a siRNA approach was employed. Transfection with Noxa siRNA reduced Noxa up-regulation but not p53 expression in HCY-treated HUVECs (Fig. 7A). Down-regulation of Noxa expression was found to significantly protect HUVECs from HCY-induced apoptosis compared with cells treated with HCY alone without siRNA transfection, whereas cells that were transfected with siRNA and not treated with HCY exhibited unchanged cell viability (Fig. 7B). These results indicate that up-regulation of Noxa plays an important role in HCY-induced apoptosis.

NO Forms S-NOHCy, Which Does Not Increase ROS Generation, p53/Noxa Expression, and Apoptotic Cell Death—Reaction of thiol-containing proteins or compounds with NO forms S-nitrosylated products and regulates the biological activity of a great variety of target proteins and compounds. It has been shown that NO can interact with HCY to form S-NOHCy, which provides cytoprotective and anti-thrombotic properties in endothelial cells by inhibiting sulphhydryl-dependent generation of ROS, ultimately leading to unopposed HCY-mediated oxidative injury (8). To examine whether NO increases the formation of S-NOHCy and suppresses intracellular ROS generation, we incubated control and AdiNOS-transfected HUVECs with HCY and measured the level of S-nitrosylated thiols by Saville’s method (Fig. 8A). HUVECs incubated with HCY resulted in a significant increase in the formation of S-nitrosylated thiols in the cultured medium compared with that of cells alone, and this increase was further enhanced by AdiNOS transfection. However, the addition of NMA inhibited the formation of S-nitrosylated compounds in both systems. These results indicated that most of the S-nitrosylated compounds may be S-NOHCy produced by the reaction of exogenous HCY and NO. To examine the effects of S-NOHCy on ROS generation, p53 and the cells were incubated with HCY regardless of iNOS expression. Under the same experimental conditions, HCY increased apoptosis of p53<sup>−/−</sup> endothelial cells by about 40% compared with minimal apoptosis (less than 15%) of p53<sup>+/+</sup> cells (Fig. 6B). The apoptotic response of p53<sup>−/−</sup> endothelial cells to HCY was significantly inhibited by AdiNOS transfection, whereas iNOS gene transfer did not nearly affect cell viability of p53<sup>−/−</sup> endothelial cells. The inhibitory effect of AdiNOS was reversed by the addition of NMA.

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dose-dependent manner (Fig. 8D). However, ROS generation, p53/Noxa expression, and cytotoxicity were not observed in HUVECs treated with 1 mM S-NOHCy alone. This suggests that NO may prevent the pathogenesis of HCY-mediated vascular diseases through the formation of S-NOHCy.

**DISCUSSION**

This study was undertaken to elucidate the cytotoxic effect and molecular mechanism of HCY on primary cultured human endothelial cells and to determine the functional role of NO and antioxidants in HCY-induced endothelial cell apoptosis. We found that HCY caused membrane lipid peroxidation, increased expression of p53 and Noxa, and caspase-dependent apoptosis of HUVECs by ROS generation. These molecular events and cytotoxicity were inhibited by NO produced from an exogenous chemical NO donor and iNOS transfer as well as ROS-depotoxifying antioxidants such as α-tocopherol and SOD plus catalase. Furthermore, HCY increased the expression of p53 and Noxa and apoptosis in endothelial cells from normal mice, but not in the cells from p53−/− mice. The cytotoxic effect of HCY was decreased by siRNA-mediated suppression of Noxa expression, indicating that Noxa up-regulation plays an important role in HCY-induced endothelial cell apoptosis. 

HCY increased the expression of p53 and Noxa, and modulated ROS generation from HCY through the formation of S-NOHCy. 

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These findings indicate that HCY caused caspase-8-independent activation of the mitochondrial apoptotic pathway, probably by ROS-dependent oxidative stress. This suggests that caspase inhibitors, antioxidants, or anti-apoptotic drugs may have a therapeutic or preventive potential for HCY-mediated atherosclerotic pathogenesis and development.

ROS and lipid peroxidation products induce DNA damage (28, 29) that can cause a specific cellular response whereby the protein product of the tumor suppressor gene p53 accumulates to high levels (30). It has been shown that p53 up-regulation and apoptotic cell death were observed in coronary and peripheral specimens retrieved from patients with primary atherosclerotic lesions (31). Recent studies have demonstrated that oxidative stress or infection of human cytomegalovirus, which plays a role in the development of atherosclerosis, induced apoptosis in human aortic endothelial cells through the up-regulation of p53 (32, 33). Up-regulation of p53 seems to be directly responsible for the regulation of several genes, such as Apaf-1, DR5, p53AIP, PUMA, and Noxa, that are proposed to mediate p53-dependent apoptosis in many types of cells (34). Noxa, the only BH3 domain-containing proapoptotic protein, has been appeared to induce the permeabilization of the mitochondrial outer membrane and the efflux of mitochondrial cytochrome c, which interacts with Apaf-1, procaspase-9, and dATP to form a complex called the apoptosome (35). This complex dimerizes and activates caspase-9, which then activates effector caspases including caspase-3 to induce apoptosis. Although HCY has induced p53 expression and caspase-dependent apoptosis in primary cultured hippocampal neurons (36), the involvement of the p53-dependent pathway has not been reported in HCY-mediated endothelial dysfunction. We hypothesized that ROS generated from HCY can activate the p53-dependent apoptotic signal pathway via oxidative stress. In this study, we examined the role of p53 as a critical mediator in HCY-induced caspase activation and apoptosis responsible for endothelial dysfunction as well as pathogenesis of vascular diseases. We first found that HCY increased the expression of p53 and its transactivated gene Noxa, leading to cytochrome c release and caspase-9 and -3 activation in HUVECs (Fig. 5). We also showed that HCY induced apoptotic cell death by increasing p53-dependent Noxa expression in mouse endothelial cells, but not in endothelial cells from p53−/− mice (Fig. 6). Furthermore, we confirmed the functional involvement of Noxa up-regulation in HCY-induced HUVEC apoptosis by knocking down Noxa expression using siRNA technology. The specific knockdown of cFLIP by transfection with Noxa siRNA effectively inhibited HCY-induced HUVEC apoptosis (Fig. 7B). These findings suggest that endothelial apoptosis by HCY may require p53-dependent Noxa expression for activation of the mitochondrial apoptotic signaling pathway. These data provide new insight into the molecular mechanism by which p53-dependent up-regulation of Noxa expression may play an important role in the pathogenesis of HCY-induced atherosclerosis.

**NO synthesized from L-arginine through the catalyzing reaction of endothelial NOS has been suggested to be the most important factor for vascular function in controlling endothelium integrity and smooth muscle contractility (13). A decrease in endothelial NO production and NO bioavailability appears to result in an impaired vascular relaxation (37) and endothelial cell dysfunction and apoptosis (38, 39), which could ultimately contribute to various vascular diseases, including atherosclerosis and thrombosis (38). The importance of NO in vascular disease has been demonstrated by findings that atherosclerotic lesions in mice deficient in endothelial NOS or administrated with NOS inhibitor developed significantly greater than control mice (40, 41). It has also been demonstrated that patients with developing atherosclerosis have reduced NO bioavailability in both the coronary and peripheral vasculature (42). The elaboration of NO, moreover, has been found to be crucial to the normal homeostatic function of the vascular endothelium (43).
This evidence suggests that an increase in NO production and bioavailability may protect endothelial cells from biological dysfunction and apoptosis in the pathogenic conditions associated with hyperhomocysteinemia. We here examined the functional role and molecular mechanism of NO in HCY-induced endothelial cell apoptosis. We found that an increase in NO production by SNAP and AdiNOS transfer inhibited apoptosis, ROS generation, lipid peroxidation, and p53/Noxa expression in HUVECs treated with HCY. These biochemical events were also inhibited by antioxidants such as α-tocopherol and SOD plus catalase, indicating that ROS is a major cytotoxic mediator for HCY-mediated endothelial cytotoxicity. The suppressive effect of NO on HCY-mediated ROS generation can be conducted by two possible mechanisms; first, NO rapidly reacts with superoxide to yield peroxynitrite, which can mediate nitrotyrosine formation as an indicator of peroxynitrite production in the vascular disease condition (44). However, we found that SNAP did not affect the formation of nitrotyrosylated proteins in HCY-treated HUVECs as measured by Western blot analysis (data not shown). This finding indicates that the alternative pathway is involved in NO-mediated suppression of ROS generation. NO interacts with HCY to form S-NHCY, which inhibits ROS generation by blocking the autoxidation of its sulfhydryl groups (45). Our data showed that AdiNOS-dependent NO production resulted in an increase in the formation of S-NHCY (Fig. 8). These data indicate that the regulation of NO production in endothelium ameliorates the potential and adverse properties of HCY in pathogenesis of hyperhomocysteinemia by the formation of S-NHCY.

Thios are critical sites of the reaction with NO for the redox-based formation of S-nitrosothiols (46). Although HCY has been known to initially decrease NO bioavailability in endothelial cells by the formation of S-NHCY, this compound can preserve the endogenous bioactivity of NO through regeneration of NO, which plays an integral role in the homeostatic process of vasculature (8, 13), and suppress ROS production by way of the thiol group (8). We defined the cytoprotective effect of S-NHCY in terms of biological and biochemical assays for ROS generation, p53/Noxa expression, and cell viability. Our data showed that S-NHCY suppressed ROS generation, p53 and Noxa expression, and apoptosis in endothelial cells treated with the cytotoxic level (1 mM) of HCY (Fig. 8), indicating that S-NHCY may inhibit the cytotoxic ROS generation from HCY by interfering with the interaction between its free sulfhydryl groups. Our data also showed that 50% of the inhibitory activity of S-NHCY for ROS generation and apoptotic cell death was detected at about 25% of the ratio between S-NHCY and HCY (Fig. 8). This indicates that the pathogenic activity of HCY may be determined by local concentration of NO in endothelium and the relative ratio of S-NHCY to HCY.

Our data provide new insight into the molecular mechanism by which HCY induces endothelial dysfunction through p53/Noxa-dependent apoptosis. We also showed that NO derived from chemical donors and NOS gene transfer protects endothelial cells from HCY-induced apoptosis by suppressing p53-dependent Noxa expression, probably via the redox-based S-nitrosylation of HCY. Thus, regulation of vascular NO production may possess therapeutic potential in atherosclerosis and thrombosis with a risk factor.
Nitric Oxide Inhibition of Homocysteine-induced Human Endothelial Cell Apoptosis by Down-regulation of p53-dependent Noxa Expression through the Formation of S-Nitrosohomocysteine

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