Homocysteine-Induced Caspase-3 Activation by Endoplasmic Reticulum Stress in Endothelial Progenitor Cells from Patients with Coronary Heart Disease and Healthy Donors

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Previous studies have suggested an association of hyperhomocysteinemia-induced vascular pathology with enhanced apoptotic potential of endothelial progenitor cells in patients with coronary heart disease. Our results indicate that 500 μmol/L homocysteine induced endothelial progenitor cell apoptosis and activation of caspase-3, both of which were abolished by 100 μmol/L and 200 μmol/L salubrinal, an agent that prevents endoplasmic reticulum stress-induced apoptosis. The addition of 500 μmol/L homocysteine caused a release of Ca^{2+} from intracellular stores, and enhanced phosphor-eukaryotic initiation factor 2α phosphorylation at Ser51 and the expression of a glucose-regulated protein of 78 kDa and a C/EBP homologous protein independently of extracellular Ca^{2+}. These effects of homocysteine on endothelial progenitor cells were significantly greater in patients with coronary heart disease than in healthy donors. These findings suggest that homocysteine induces endoplasmic reticulum stress-mediated activation of caspase-3 in endothelial progenitor cells, an event that is enhanced in patients with coronary heart disease. Furthermore, enhanced endoplasmic reticulum stress-mediated activation of caspase-3 in endothelial progenitor cells might be involved in hyperhomocysteinemia-associated vascular pathology.

Key words: homocysteine; endothelial progenitor cells; apoptosis; endoplasmic reticulum stress

It is generally recognized that hyperhomocysteinemia (HHcy) is an important risk factor for coronary artery disease (CAD), but the pathogenesis of its vascular action remains to be elucidated. Accumulating evidence has demonstrated that endothelial dysfunction plays a major role in HHcy-related vascular pathology. Endothelial dysfunction is a loss of balance between injury and repair capacity. Recent studies suggest an important role of endothelial progenitor cells (EPCs) in endothelial repair after injury. CAD Patients have decreased numbers of EPCs with impaired functional capacity. In vitro, homocysteine (Hcy) dose- and time-dependently decreases EPC numbers and impairs cell proliferation, migration, and vasculogenesis capacity. Impaired EPC numbers and functional capacity have been identified in a murine model with HHcy. One possible explanation for the reduced capacity of EPCs is increased apoptosis of premature progenitor cells. Recently, Bao found that Hcy induces apoptosis of EPCs in vitro. Taken together, these findings suggest an association of Hcy with the enhanced apoptotic potential of EPCs in CAD patients.

The endoplasmic reticulum (ER) has a high sensitivity to alteration in Ca^{2+} homeostasis, and a low luminal Ca^{2+} concentration ([Ca^{2+}]_{ER}) leads to oxidative stress and the accumulation of misfolded or unfolded proteins, resulting in ER dysfunction and stress. ER stress is triggered by an accumulation of misfolded proteins in the ER that bind to an ER chaperone glucose-regulated protein of 78 kDa (Grp78). This causes the unfolded protein response (UPR), resulting in the dissociation of Grp78 from the three major ER stress sensors: protein kinase RNA-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring protein-1 (IRE-1). PERK-dependent phosphorylation of eukaryotic initiation factor 2α (eIF2α) results in translational attenuation, reducing the load of new protein synthesis on the ER. These responses re-establish homeostasis and normal ER function. If ER stress is severe or prolonged, apoptotic cell death is induced. Several apoptosis pathways are known to be involved, the central role being played by the proapoptotic transcription factor C/EBP homologous protein (CHOP). Transcriptional induction of CHOP depends mostly on activation of PERK/eIF2α. ER stress has been found to be involved in endothelial dysfunction and related vascular pathologies. Upregulation of Grp78 and other ER stress-related genes was detected in a murine model of myocardial infarction. Both cytoprotective and apoptotic components of ER stress signaling are upregulated in heart failure. High levels of apoptosis and of the expression of Grp78 and CHOP were found within fibrous caps of atheroma and ruptured plaques of both autoimmune human coronary specimens and atherectomy specimens from patients with unstable angina pectoris. A previous report provided evidence of the relevance of ER stress to macrophage apoptosis and enlargement of the necrotic core in advanced athero-

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sclerotic plaques.\(^{11}\) Although there are no reports regarding ER stress in EPCs, other resources of adult stem and progenitor cells, such as mesenchymal stem cells, have been reported to be induced to apoptosis via the ER pathway.\(^{12}\) Hence, we hypothesized that there is ER stress-induced apoptosis in EPCs.

Hcy is a well-known inducer of ER stress. Apolipoprotein E-deficient mice fed a HHCy diet exhibit increased expression of molecular markers of ER stress in both early and late atherosclerotic lesions.\(^{13}\) In a rat model of HHCy, increased expression of ER stress-associated proteins, including Grp78, CHOP, and caspase-12, was found in myocardial tissue.\(^{14}\) In vitro, Hcy induces ER stress and cell apoptosis in cardiomyocytes,\(^{14}\) smooth muscle cells,\(^{15}\) and endothelial cells.\(^{16}\) Although these studies indicate that Hcy is closely associated with ER stress-mediated cell apoptosis, it is yet not clear whether ER stress contributes to HHcy-related vascular pathology, or how this might occur. Hence, we investigated the possible role of Hcy in ER stress-mediated apoptosis in EPCs from CAD patients and healthy donors in an attempt to elucidate the mechanisms underlying HHcy-related vascular pathology associated with CAD.

**Materials and Methods**

**Materials.** EGM-2 endothelial medium and EGM-2-MV-Single-Quots were purchased from Clonetics (San Diego, CA). Fura-2 AM, fura-FF AM, plumeric acid, and plumeric acid were from Molecular Probes (Leiden, Netherlands). Hcy, fibronectin (FN), thapsigargin (TG), ionomycin, 1,2-bis(o-aminophenoxylethene-N,N,Nˊ,Nˊ-tetraacetic acid (BATPA), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Sigma-Aldrich (St. Louis, MO). Salubrinal was from Tocris (Bristol, UK). Anti-Grp78 and -phospho-eIF2α (Ser51) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents are indicated below.

**Subjects.** Patients with CAD were recruited from the Cardiovascular Disease Clinic and Ward at Zhejiang Hospital (Zhejiang, China). A diagnosis of CAD was reached by coronary angiography. Patients with malignant disease, peripheral vascular disease, proliferative retinopathy, or recent (<2 months) acute coronary syndrome, inflammation, bleeding, or blood transfusion were excluded, since these conditions and procedures might have affected the number or function of EPCs. None of the patients had been treated with statins or erythropoietin in the preceding 2 months. The control subjects were age- and sex-matched healthy volunteers. This study was approved by the Research Ethics Review Board of Zhejiang Hospital and complied with the Code of Ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002). Informed consent was obtained in writing from all subjects.

**Isolation, incubation, and identification of EPCs.** EPCs were prepared as described previously.\(^{17}\) Briefly, mononuclear cells were isolated from peripheral blood by Ficoll density gradient centrifugation and cultured on human FN-coated dishes in EGM-2 endothelial medium supplemented with EGM-2-MV-SingleQuots and 20% FBS.

**Cellular apoptosis detected by flow cytometer.** EPCs were seeded and pre-cultured at 37 °C for 24 h. After treatment with various drugs for various durations, EPCs were collected and treated according to the protocol in the Annexin V-FITC Apoptosis Detection Kit (Merck, Germany). The percentages of apoptotic cells were determined with a FACS Calibur flow cytometer with CellQuest software (BD Pharmingen, San Diego, CA).

**Measurement of caspase-3 activity.** The activity of caspase-3 was evaluated using a colorimetric activity assay kit (Chemicon Internacional Molecular, Temecula, CA) following the manufacturer’s instructions. Briefly, the cells were lysed for 30 min on an ice bath. The lysed cells were centrifuged at 12,000 x g for 10 min. After the protein concentration was determined, 100 μg of the protein was incubated with 50 μL of a reaction buffer and 5 μL of the caspase-3 substrate (Ac-DEVD-pNa) on a 96-well plate. The activity of caspase-3 was measured using a spectrophotometer at 405 nm, and the results were expressed as fold increase over mean value of the control.

**Measurement of the intracellular free calcium concentration ([Ca^2+]).** To monitor [Ca^2+]i, cells were incubated at 37 °C with 2 μmol/L of fura-2 AM for 45 min. Fluorescence was recorded from 1 mL aliquots of magnetically stirred cell suspensions (106 cells/mL) at 37 °C using a spectrophotometer (Zeiss, Jena, Germany) with λ ex 340 and 380 nm and λ em 505 nm. Changes in [Ca^2+]i were monitored using the fura-2 340/380 fluorescence ratio and calibrated. Ca^2+ release was estimated using the integral of the rise in [Ca^2+]i for 2.5 min after the addition of agents.

**Measurement of the free calcium concentration in the intracellular stores ([Ca^2+]s).** Endothelial progenitor cells were incubated with 5 μmol/L of fura-FF AM for 1 h in the presence of 0.025% plumeric acid, and were loaded with 10 μmol/L dimethyl BATPA for 30 min. EPCs were then collected by centrifugation at 1,000 x g for 10 min and resuspended in HEPES-buffered saline. Fluorescence was recorded from 1 mL aliquots of magnetically stirred cell suspensions (106 cells/mL) at 37 °C using a spectrophotometer (Zeiss) with λ ex 340 nm and λ em 505 nm. Changes in [Ca^2+]s were expressed as F0/F1, considering F0 as fluorescence of fura-FF in the absence of free Ca^2+.

**Immunoblotting.** Following treatment, whole-cell lysates were prepared as described previously.\(^{17}\) In some experiments, immunoblotting for CHOP protein was additionally performed on nuclear extracts prepared using a Nuclear Extraction Kit following the manufacturer’s protocol (Millipore, Billerica, MA). The protein concentration was determined by the Lowry method. Ten μL of protein was loaded in each lane and subjected to SDS-PAGE (8%), and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were probed with antibodies against phospho-eIF2α (Ser51) and GAPDH. Horseradish peroxidase-conjugated secondary IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Statistical analysis.** All data are presented as mean ± SEM. Statistical evaluation of the data was performed by Student’s t test for paired or unpaired observations and by analysis of variance (ANOVA). Scheffé’s test for multiple comparisons was used to identify differences among groups. Values were considered to be significantly different at p < 0.05.

**Results**

**Comparison of the numbers of EPCs from CAD patients and healthy donors.** Approximately 5 × 10⁶ mononuclear cells from every CAD patient and healthy control were seeded. The number of EPCs was significantly higher (0.064 ± 0.004) in the CAD patients than in the healthy controls (0.032 ± 0.004; p < 0.01, n = 12 in each group) (Fig. 1).

**Comparison of apoptosis of EPCs induced by Hcy from CAD patients and healthy donors.** The apoptosis of EPCs induced by Hcy was examined in the presence of various concentrations of Hcy (0, 50, 100, and 500 μmol/L) over 24 h and in the presence of 500 μmol/L Hcy for 0, 12 h, 24 h, and 48 h. As shown in
Fig. 2. After 24 h Hcy induced a detectable increase in the percentage of apoptotic EPCs with 100 μmol/L to 3.8 ± 0.8% and 5.1 ± 0.9% in healthy donors and CAD patients respectively, with a maximal effect at 500 μmol/L (7.4 ± 1.2% vs. 8.2 ± 0.8%, p < 0.05), and also after 48 h of treatment (13.7 ± 1.3% vs. 15.9 ± 0.9%, p < 0.05) in healthy donors and CAD patients.

Hcy induced ER-dependent caspase-3 activation in EPCs from CAD patients and healthy donors

Caspase-3 activation of EPCs induced by Hcy was examined in the presence of various concentrations of Hcy (0, 50, 100, and 500 μmol/L) for 24 h and in the presence of 500 μmol/L Hcy for 0, 12 h, 24 h, and 48 h. Hcy induced caspase-3 activity in a dose- and time-dependent manner in healthy donors and CAD patients, with a maximal effect at 500 μmol/L (3.8 ± 0.5- and 4.7 ± 0.4-fold increases vs. control untreated cells; p < 0.05, n = 6 in each group) and after 48 h of treatment (4.8 ± 0.5- and 5.7 ± 0.4-fold increases vs. untreated cells; p < 0.05, n = 6 in each group) (Fig. 3A and B).

To determine whether the caspase-3 activation induced by Hcy is a result of endoplasmic reticulum stress, we used salubrinal, a cell-permeant, selective inhibitor
of eIF2α dephosphorylation that protects cells from endoplasmic reticulum stress. EPCs from healthy donors and CAD donors were stimulated with Hcy (500 μmol/L) after pretreatment with various concentrations of salubrinal (10, 100, 200 μmol/L) for 30 min. The Hcy-evoked increase in caspase-3 activity was inhibited by pretreatment with salubrinal in both CAD patients and healthy donors, with a maximum effect at 200 μmol/L, with a 1.3 ± 0.5- and 1.6 ± 0.4-fold increases vs. control (untreated cells) in healthy donors and CAD patients respectively (p < 0.05, n = 6 in each group) (Fig. 3C). Figure 3D shows that salubrinal inhibited Hcy-induced apoptosis of EPCs from healthy donors and CAD patients.

**Hcy mobilized Ca²⁺ from intracellular pools**

The intracellular free calcium concentration and \([Ca^{2+}]_{ER}\) were measured in the presence of 50, 100, and 500 μmol/L Hcy. In a Ca²⁺-free medium, treatment of fura-2-loaded EPCs with Hcy induced a dose-dependent increase in cytosolic Ca²⁺ due to Ca²⁺ release from intracellular Ca²⁺ compartments. Cytosolic Ca²⁺ concentrations, both at rest and after stimulation with Hcy, were significantly greater in CAD patients than in healthy donors (Fig. 4A–D). To investigate further the ability of Hcy to release Ca²⁺ from intracellular stores, EPCs were loaded with fura-FF, a common tool to measure compartmentalized Ca²⁺. In addition, cells were loaded with dimethyl BAPTA and suspended in a Ca²⁺-free medium (100 μmol/L EGTA added) to avoid interference with cytosolic and extracellular Ca²⁺ respectively. As shown in Fig. 4E, Hcy (0, 50, 100, and 500 μmol/L) decreased \([Ca^{2+}]_{ER}\) due to Ca²⁺ release in a dose-dependent manner. The Ca²⁺ remaining in the stores after 10 min of treatment with Hcy was estimated by adding a combination of thapsigargin and ionomycin to deplete the intracellular Ca²⁺ stores fully. Values are mean ± SEM. *p < 0.05 as compared with untreated cells.

![Fig. 4. Hcy Mobilized Ca²⁺ from Intracellular Pools in EPCs.](image)
Hcy induced UPR activation in EPCs from CAD patients and healthy donors

Since Hcy induces Ca\(^{2+}\) efflux from intracellular stores, which can induce ER stress, UPR activation was detected by immunoblotting using specific anti-phospho-eIF2\(\alpha\) and anti-Grp78 antibodies. As shown in Fig. 5A and B, in a Ca\(^{2+}\)-free medium, Hcy (500 \(\mu\)mol/L) induced time-dependent phosphorylation of eIF2\(\alpha\) and expression of Grp78. These were significantly greater in the EPCs from the CAD patients than from the healthy donors. The maximal effect of Hcy was obtained after 48 h of treatment in CAD patients and healthy donors. In a medium containing 1 mmol/L of Ca\(^{2+}\), similar results were obtained for the phosphorylation of eIF2\(\alpha\) and the expression of Grp78 (data not shown).

Since prolonged or severe ER stress is linked to the triggering of programmed cell death, the effect of Hcy on CHOP, a major ER stress-inducible pro-apoptotic transcription factor that operates as a downstream component of the ER-stress pathway, was investigated. We found that CHOP expression increased after treatment with Hcy (500 \(\mu\)mol/L) for various times (0, 12, 24, and 48 h). Maximum CHOP expression was obtained at 48 h of exposure to Hcy (Fig. 5C). In a medium containing 1 mmol/L of Ca\(^{2+}\), similar results were obtained for the expression of CHOP (data not shown).

Discussion

In this study, we confirmed the observations of Bao\(^7\) and Alam,\(^19\) who reported that Hcy induces apoptosis of peripheral EPCs in vitro. We report for the first time that the Hcy-induced apoptotic potential of EPCs from patients with CAD was higher than that from healthy donors. Furthermore, Hcy-induced EPC apoptosis was operational via ER stress-mediated caspase-3 activation.

Patients with CAD show decreased numbers of EPCs with impaired functions, which can be due to several mechanisms such as calcium signaling and oxidative stress. The imbalance between oxygen radical generation and antioxidant power plays an important role in the pathogenesis of Hcy-related vascular pathology, and oxidative stress has been found to be involved in the development of vascular and blood cell apoptosis, including EPCs.\(^7\) We report for the first time that Hcy induced ER stress in EPCs, independently of extracellular Ca\(^{2+}\) levels, by promoting Ca\(^{2+}\) efflux from intracellular stores. The mechanism underlying Ca\(^{2+}\) release from intracellular stores by Hcy was not further investigated, but it is probably attributable to the oxidant actions of Hcy. Oxygen radicals have been reported to induce Ca\(^{2+}\) release in various cell types, including endothelial cells,\(^20\) smooth muscle cells,\(^21\) and cardiomyocytes.\(^22\)
In our study, Hcy enhanced phosphorylation of eIF2α at Ser, and increased the expression of Grp78 and CHOP in a time-dependent manner. Other ER stress activators, such as TG can increase Grp78 and CHOP, confirming the ability of Hcy to induce ER stress in EPCs. ER stress has been suggested to lead to the activation of certain caspases, including caspase-3, caspase-4, and caspase-9. We found that Hcy caused time-dependent activation of caspase-3. The activation of caspase-3 is probably attributable to ER stress, since salubrinal, a cell-permeant inhibitor of eIF2α phosphatase at Ser, prevented the caspase-3 activation induced by Hcy. However, another mechanism might be involved in Hcy-induced caspase-3 activation. Alam et al. reported that Hcy induces apoptosis in EPCs through the mitochondrial pathway.

We found that high concentrations of Hcy induced greater survival/rescue molecules, phosphorylated eIF2α and Grp78 expression, and apoptotic molecules, and enhanced CHOP and activated caspase-3 expression in peripheral EPCs from CAD patients. These findings might be explained by a loss of efficiency of the UPR due to UPR dysfunction induced by Hcy. UPR dysfunction has been found in a number of disorders, including non-alcoholic fatty liver disease, renal disorders, and diabetes mellitus. Additional evidence suggests that ER stress and UPR dysfunction play important roles in the pathogenesis of atherosclerosis, contributing to endothelium dysfunction. Here we report for the first time that there was UPR dysfunction in CAD. Our finding that Hcy-induced EPC actions, including Ca2+ mobilization, eIF2α phosphorylation, Grp78 and CHOP expression, and caspase-3 activation, were significantly greater in patients with CAD than in healthy donors, suggests that Hcy is more efficient at inducing EPC effects in CAD patients than in healthy donors. Taken together with the reported higher Hcy levels in CAD patients, these findings suggest that plasma Hcy plays an important role in the pathogenesis of HHcy-related vascular pathology. Our results indicate that ER stress, and subsequently eIF2α phosphorylation and Grp78 and CHOP expression, were similar in EPCs from healthy donors and CAD patients at rest. This might be because, despite the finding that resting [Ca2+]i in EPCs from CAD patients was enhanced as compared with healthy controls, the amount of Ca2+ accumulated in the stores was comparable in the EPCs from CAD patients and healthy donors. Thus, the ER stress level at rest was comparable in the EPCs from CAD patients and healthy donors.

In summary, our results indicate for the first time that the Hcy-induced apoptotic potential of EPCs from CAD patients is greater than that from healthy donors. Furthermore, Hcy-induced EPCs apoptosis is operational via ER stress-induced caspase-3 activation. The EPCs from CAD patients showed a higher sensitivity to Hcy than those from healthy donors. These findings provide evidence of a new mechanism underlying Hcy-induced apoptosis that might be involved in the pathogenesis of HHcy-related vascular pathology.

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