Alterations in the cellular redox potential by homocysteine promote endothelial cell (EC) dysfunction, an early event in the progression of atherothrombotic disease. In this study, we demonstrate that homocysteine causes endoplasmic reticulum (ER) stress and growth arrest in human umbilical vein endothelial cells (HUVEC). To determine if these effects reflect specific changes in gene expression, cDNA microarrays were screened using radiolabeled cDNA probes generated from mRNA derived from HUVEC, cultured in the absence or presence of homocysteine. Good correlation was observed between expression profiles determined by this method and by Northern blotting. Consistent with its adverse effects on the ER, homocysteine alters the expression of genes sensitive to ER stress (ie, GADD45, GADD153, ATF-4, YY1). Several other genes observed to be differentiated by homocysteine are known to mediate cell growth and differentiation (ie, GADD45, GADD153, Id-1, cyclin D1, FRA-2), a finding that supports the observation that homocysteine causes a dose-dependent decrease in DNA synthesis in HUVEC. Additional gene profiles also show that homocysteine decreases cellular antioxidant potential (glutathione peroxidase, NKEF-B PAG, superoxide dismutase, clusterin), which could potentially enhance the cytotoxic effects of agents or conditions known to cause oxidative damage. These results successfully demonstrate the use of cDNA microarrays in identifying homocysteine-responsive genes and indicate that homocysteine-induced ER stress and growth arrest reflect specific changes in gene expression in human vascular EC.

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Hyperhomocyst(e)inemia (HH) is a significant independent risk factor for premature atherothrombotic disease. Up to 40% of patients diagnosed with coronary, cerebrovascular, or peripheral atherosclerosis have HH. Although the majority of cases of HH are thought to be caused by an interplay between dietary and genetic factors, the genetic disorders are associated with the highest plasma levels of homocysteine, with cystathionine β-synthase deficiency (CBS) and 5,10-methylenetetrahydrofolate reductase deficiency being the most common. Regardless of the underlying cause of HH, the relationship between elevated blood homocysteine levels and premature vascular and thrombotic disease persists.

Earlier studies suggest that atherothrombosis associated with HH reflects endothelial cell (EC) injury and/or dysfunction. Homocysteine causes EC injury when administered to baboons or rats, or when added directly to cultured EC. Furthermore, deGroot et al showed that primary cultures of EC obtained from obligate heterozygotes for CBS deficiency are more sensitive to homocysteine-induced damage than control cells. In addition to causing injury, homocysteine has been shown to increase the procoagulant activity of cultured EC by (1) inducing a protease that activates factor V, (2) inhibiting protein C activation, (3) causing aberrant processing of thrombomodulin (TM), (4) inducing tissue factor activity, and (5) inhibiting the cellular binding sites for tissue plasminogen activator. Homocysteine also reduces nitric oxide production in vitro, a finding that could explain why diet-induced HH in monkeys and pigs causes impaired vasomotor regulatory function.

Currently, the mechanism by which elevated levels of homocysteine cause EC injury and/or dysfunction is relatively unknown. We, and others, have demonstrated in cultured human vascular EC that homocysteine increases the expression and synthesis of GRP78, an endoplasmic reticulum (ER)-resident chaperon and member of the 70-Kd heat-shock protein (HSP) family. In support of these in vitro findings, steady-state mRNA levels of GRP78 were also shown to be elevated in the livers of CBS-deficient mice that had HH. Given that

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MATERIALS AND METHODS

Cell culture and treatment conditions. Primary human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment of human umbilical veins and cultured in EC medium (M199 medium containing 20% fetal bovine serum, 20 µg/mL EC growth factor, 90 µg/mL porcine intestinal heparin, 100 µg/mL penicillin, and 100 µg/mL streptomycin) in a humidified incubator at 37°C with 5% CO₂. Cells from passages 2 to 4 were used in these studies. The transformed HUVEC line, ECV304, was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in EC medium. DL-Homocysteine or dithiothreitol (DTT) (Sigma, St Louis, MO) was prepared in EC medium, sterilized by filtration, and added to the cell cultures.

\[^{3}H\]Thymidine incorporation. HUVEC were grown to 70% confluence in EC medium in the absence or presence of increasing concentrations of homocysteine or DTT (0.2 to 5.0 mmol/L) for 18 hours. After the last 4 hours of treatment, cells were labeled with \[^{3}H\]thymidine (NEN Life Sciences, Guelph, Canada) at 1 µCi/mL. After labeling, cells were washed 3 times with phosphate-buffered saline (PBS), fixed in ice-cold 10% acetic acid, and washed with 95% ethanol. Samples with ratios greater than 1.6 were stored at −70°C for further analysis.

Effect of homocysteine on HUVEC viability and growth. To examine the effect of homocysteine on cell viability, \[^{51}Cr\] release assays were performed as described previously. Exposure of HUVEC to 5 mmol/L homocysteine for up to 18 hours had no significant effect on overall cell viability, compared with control cells (19.3% ± 0.5% vs 17.4% ± 0.6% release, respectively, at 6 hours, n = 3; 27.9% ± 2.3% vs 28.1% ± 0.7% release, respectively, at 18 hours, n = 3). The time-dependent basal release of \[^{51}Cr\] from HUVEC is consistent with previous studies. In contrast to 5 mmol/L homocysteine alone, 5 mmol/L homocysteine in the presence of 4 µmol/L Cu²⁺, which is known to generate H₂O₂ and induce EC lysis, caused a significant increase (P < .01) in the release of \[^{51}Cr\] at 18 hours (28.1% ± 0.7% vs 81.4% ± 1.2% release, respectively, n = 3).

To determine the effect of homocysteine on cell proliferation,
HUEVC were cultured in the absence or presence of various concentrations of homocysteine and [3H]thymidine incorporation was measured. As shown in Fig 1, HUEVC exposed to homocysteine for 18 hours demonstrated a dose-dependent decrease in DNA synthesis and is consistent with earlier studies. In addition to homocysteine, the thiol-containing reducing agent DTT also caused a similar dose-dependent decrease in DNA synthesis (data not shown).

**Homocysteine induces ER stress in HUEVC.** Previous studies have shown that intracellular transport of vWF and TM via the ER is selectively inhibited by homocysteine in HUEVC. Homocysteine also increases the expression and synthesis of GRP78, a resident ER chaperon induced by agents or conditions known to adversely affect ER function. To further investigate the effect of homocysteine on ER function, vWF processing and secretion, and its interaction with GRP78 were examined in HUEVC cultured in the absence or presence of homocysteine.

The effect of homocysteine on intracellular levels and distribution of GRP78 and vWF in HUEVC was examined by indirect immunofluorescence using anti-vWF or anti-GRP78 antibodies. In control HUEVC, GRP78 was concentrated in the perinuclear region, consistent with its presence in the ER (Fig 2A). In contrast, both the distribution and intensity of GRP78 immunostaining was markedly enhanced in HUEVC exposed to homocysteine (Fig 2B). Unlike GRP78, vWF immunostaining and localization were dramatically reduced in HUEVC exposed to homocysteine (Fig 2E), compared with control cells (Fig 2D). As controls, no specific staining was observed in untreated HUEVC incubated with either normal mouse (Fig 2C) or rabbit IgG (Fig 2F). Consistent with these findings, intracellular levels...
of vWF dimers were dramatically decreased after 4 hours in HUVEC exposed to 5 mmol/L homocysteine (Fig 3).

Stable association of GRP78 with misfolded, improperly glycosylated, or incompletely assembled proteins in the ER leads to retention and intracellular degradation. To determine whether GRP78 was capable of stably binding to misfolded vWF within the ER, HUVEC exposed to various concentrations of homocysteine for 8 or 18 hours were metabolically labeled with [35S]methionine and [35S]cysteine, and vWF from cell lysates was immunoprecipitated with anti-vWF antibodies as described in the Methods. Bands that corresponded to mature and pro-vWF were detected in immunoprecipitated eluates from HUVEC treated without or with homocysteine (Fig 4). However, coimmunoprecipitation of vWF with GRP78 was observed only in HUVEC treated with 1 or 5 mmol/L homocysteine for 8 hours or 5 mmol/L homocysteine for 18 hours. Taken together, these findings imply that GRP78 binds to misfolded vWF, prevents its secretion from the ER and likely directs aberrantly folded vWF to the degradative machinery.

Effect of homocysteine on differential gene expression in HUVEC. Previous studies have shown that cDNA arrays provide a rapid and effective method in monitoring differential gene expression. To investigate the possibility that homocysteine-induced ER stress and growth arrest reflect specific changes in gene expression in ECs, a human cDNA microarray that contained 588 known human genes was screened using radiolabeled cDNA generated from total poly (A) RNA from primary HUVEC cultured in the absence or presence of 5 mmol/L homocysteine for 4 or 18 hours. After hybridization, the cDNA array membranes were washed under high stringency and the hybridization patterns analyzed by autoradiography. The level of nonspecific hybridization was low since the negative DNA controls, including M13mp18(+) strand DNA, λDNA, and pUC18, failed to show any hybridization signal. To ensure accurate comparisons in the expression levels of each gene on the cDNA array, hybridization signals were normalized to the signals obtained from housekeeping gene controls (ie, ubiquitin, glyceraldehyde 3-phosphate dehydrogenase, α-tubulin, human leukocyte antigen [HLA] class I histocompatibility antigen C-4, β-actin, 23-Kd highly basic protein, ribosomal protein S9) on the same array.

As shown in Fig 5, the hybridization patterns between wild-type and homocysteine-treated HUVEC were similar. However, analysis of the cDNA microarray showed that a total of 16 of 588 (2.7%) of the known human genes were differentially expressed in primary HUVEC exposed to 5 mmol/L homocysteine for 4 or 18 hours (Table 1). Similar changes in gene expression were also observed in the ECV304 cell line cultured in the absence or presence of homocysteine (data not shown). The percentage change in gene expression observed in this study is consistent with the recent observation that 2.5% of genes are regulated in HepG2 hepatoma cells exposed to β-mercaptoethanol, another thiol-containing agent known to cause ER stress and growth arrest. Of these genes, 9 were shown to be induced by homocysteine, while 7 were downregulated. Among the genes detected with significantly higher expression (>10-fold) was GADD153, a stress-response gene known to be induced by agents or conditions that adversely affect ER function. ATF-4, a stress-inducible transcription factor, was also shown to be induced by homocysteine and is consistent with earlier studies using mRNA differential display to identify ATF-4 as a homocysteine-inducible gene. Other inducible genes included Id-1, guanine nucleotide-binding protein G-S, SREBP, transcriptional repressor protein YY1, and the transcription factor ETR103. Genes shown to be downregulated by homocysteine included the antioxidant enzymes NKEF-B, superoxide dismutase, glutathione peroxidase, clusterin, and PAG, as well as FRA-2, adenosine triphosphate (ATP)-dependent DNA helicase, and cyclin D1. The observation that the majority of significant changes in gene expression occurred at 4 hours and declined by 18 hours suggests that homocysteine causes an initial early response in EC gene expression, followed by an adaptive response that likely involves cellular factors that influence the metabolism or elimination of intracellular homocysteine.
To test the reliability of the cDNA microarrays in identifying differentially expressed genes, we analyzed 7 different homocysteine-responsive genes by Northern blot analysis (Fig 6A). In each case, the relative expression levels of these homocysteine-responsive genes observed on Northern blots was consistent with the differential gene expression identified by microarray hybridization (Table 2). As a positive control, homocysteine was shown to induce the expression of GRP78 (Fig 6B), a finding consistent with our earlier studies.  

**DISCUSSION**

Previous studies using cultured human vascular EC have shown that alterations in the cellular redox potential by homocysteine impair protein processing and secretion via the

<table>
<thead>
<tr>
<th>5 mmol/L Homocysteine, 4 h</th>
<th>Relative Change in Gene Expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADD153†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADD45†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATF-4†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA-binding protein inhibitor Id-1†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterol regulatory element binding protein (SREBP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanine nucleotide-binding protein G-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YY1†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fos-related antigen 2 (FRA-2)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural killer-enhancing factor β (NKEFB)§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation-associated glycoprotein (PAG)§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1 (G1/S-specific)‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clusterin§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP-dependent DNA helicase</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>5 mmol/L homocysteine, 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADD153</td>
</tr>
<tr>
<td>GADD45</td>
</tr>
<tr>
<td>ATF-4</td>
</tr>
<tr>
<td>SREBP</td>
</tr>
<tr>
<td>Guanine nucleotide-binding protein G-5</td>
</tr>
<tr>
<td>Transcription factor</td>
</tr>
<tr>
<td>ETR103</td>
</tr>
<tr>
<td>Superoxide dismutase§</td>
</tr>
<tr>
<td>ATP-dependent DNA helicase</td>
</tr>
</tbody>
</table>

*Relative gene expression levels were determined by normalizing the hybridization signals to the signals obtained from the housekeeping genes included on the cDNA array. Results represent the averages of 2 independent hybridization experiments. ||| >10-fold induction v wild type; |||, 5- to 10-fold induction v wild type; ||, 2- to 5-fold induction v wild type; ||, >10-fold downregulation v wild type; ||, 5- to 10-fold downregulation v wild type; ||, 2- to 5-fold downregulation v wild type. †ER stress response genes. ‡Cell growth and differentiation genes. §Oxidative stress response genes.
ER 18, 28 and increase expression of the ER stress-response gene, GRP78. 24-26 In this study, we demonstrate that homocysteine causes ER stress in cultured human vascular EC by (1) decreasing intracellular levels of vWF, (2) inducing the expression and synthesis of GRP78, and (3) increasing the stable association of vWF with GRP78. Furthermore, homocysteine was shown to cause a dose-dependent decrease in DNA synthesis, a result consistent with earlier findings.29 Taken together, these findings both support and extend previous studies using cultured vascular EC18, 24-26, 28 and suggest that homocysteine acts intracellularly by altering the cellular redox state, thereby leading to ER stress and growth arrest in EC.

To determine if homocysteine-induced ER stress and growth arrest results in specific changes in gene expression in EC, cDNA microarrays were screened. This approach was taken based on previous studies showing that cDNA microarrays provide a powerful approach for studying differential gene expression associated with the pathogenesis of cancer and other diseases.34-38 As a result of this analysis, we demonstrate that the effects of homocysteine on ER function and cell growth reflect specific changes in gene expression. Furthermore, additional gene profiles indicate that homocysteine suppresses the ability of EC to protect themselves from agents or conditions known to elicit oxidative damage.

Although relatively high concentrations of homocysteine (≥1 mmol/L) were used to evaluate changes in gene expression, there was no effect on overall cell viability, a finding consistent with previous studies that indicated EC from normal individuals are relatively resistant to high doses of homocysteine.12-19 This may reflect the fact that only a small percentage of exogenous homocysteine (<1%) added to the culture medium is actually taken up intracellularly.41 Indeed, we have shown that intracellular concentrations of homocysteine are increased only 2-fold and 5-fold in HUVEC exposed to 1 or 5 mmol/L homocysteine, respectively, compared with untreated cells.26 Thus, the high doses required to alter gene expression in vitro likely reflects the need to increase intracellular levels of homocysteine by overcoming the cellular factors that influence the metabolism and/or elimination of homocysteine.

The observation that homocysteine induces the expression of GADD45, GADD153, ATF-4, and YY1 provides genetic evidence that homocysteine causes ER stress. Although GADD45, a downstream effector of p53, and GADD153, a member of the C/EBP gene family of transcription factors,42 have also been shown to be induced by growth arrest, by DNA damage, or by UV irradiation,43-45 recent studies indicate that inducers of ER stress can also cause these responses.

Table 2. Relative Changes in Gene Expression on Northern Blots

<table>
<thead>
<tr>
<th>Human Gene</th>
<th>1 mmol/L Homocysteine</th>
<th>5 mmol/L Homocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>18 h</td>
</tr>
<tr>
<td>GADD153</td>
<td>3.4</td>
<td>4.2</td>
</tr>
<tr>
<td>GADD45</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Id-1</td>
<td>7.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Clusterin</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>NKEF-B</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>PAG</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>SREBP</td>
<td>1.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Total cellular RNA from HUVEC treatment without or with homocysteine was isolated and Northern blot analysis was performed using 10 µg of RNA per lane as described in Fig 6 and in the Methods. Following autoradiography, signal density of each RNA band, normalized to GAPDH, was expressed as relative fold-change in gene expression vs control levels. Results represent the average of 2 independent hybridization experiments.
GRP78 increase the expression of these genes,46 and that the induction of GADD153 is more responsive to ER stress.40 Based on previous studies demonstrating that DTT, a thiol-containing agent known to cause ER stress, induces the expression of both GRP78 and GADD153,27 our findings are not unexpected. Although the physiologic significance of GADD gene induction by homocysteine has not been defined, overexpression of the GADDs causes growth arrest in several cell types.48,49 Furthermore, the importance of GADD153 in cellular growth and differentiation comes from the molecular analysis of human sarcomas wherein the rearrangement of the GADD153 gene gives rise to a naturally occurring altered form of GADD153 incapable of eliciting growth arrest.50 Given our findings, as in other studies,29 that homocysteine inhibits EC growth, the GADDs may play a potential role in linking ER stress to alterations in cell growth and proliferation. This concept is also supported by our observation that DTT, a known inducer of ER stress,27 also decreases EC proliferation. The homocysteine-induced expression of ATF-4, a member of the activating transcription factor/cyclic adenosine monophosphate (cAMP)-responsive element-binding protein (ATF/CREB) family of transcription factors, is consistent with previous studies.24 ATF-4 is induced by increased intracellular Ca\(^{2+}\) concentrations51 and by anoxia,52 conditions known to alter ER function. YY1, a member of the GLI zinc finger family, has been shown to specifically enhance the transcriptional activation of the GRP78 promoter under a variety of ER stress conditions.53 These include depletion of ER Ca\(^{2+}\) stores, inhibition of glycosylation and formation of misfolded proteins. Thus, the ability homocysteine to increase the expression of YY1 would not only enhance the expression of GRP78, but could potentially mediate stress signals generated from the ER to the nucleus. Given that YY1 affects cell growth54 and acts as a transcriptional repressor,55 the induction of YY1, like the GADD genes, by homocysteine may also play a role in mediating EC growth.

Alterations in the expression of genes known to mediate cell growth and differentiation is consistent with the finding that homocysteine causes a dose-dependent decrease in EC growth. Overexpression of Id-1, a member of the helix-loop-helix transcriptional regulators,56 suppresses cell differentiation in mammary epithelial cells57 and murine erythroleukemia cells.58 The ability of homocysteine to decrease cyclin D1, a positive growth regulator during the early G\(_1\) phase,27 and FRA-2, a transcription factor known to promote osteoblast differentiation,29 also suggests that homocysteine affects the expression of growth response genes in ECs. Whether a decrease in cell growth plays a role in protecting EC from homocysteine-induced injury and/or dysfunction is currently unknown.

The ability of homocysteine to inhibit the expression of the antioxidant enzymes glutathione peroxidase and NKEF-B are consistent with our previous findings29 and suggest that homocysteine may promote EC dysfunction and/or injury by indirectly enhancing the cytotoxic effect of agents or conditions that cause oxidative stress. This concept is further supported by the recent observation that homocysteine impairs the ability of glutathione peroxidase to detoxify peroxides40 and acts synergistically with H\(_2\)O\(_2\) to enhance mitochondrial damage.55 In addition to these antioxidant enzymes, homocysteine decreased the expression of clusterin and PAG. Clusterin, a multifunctional heterodimeric glycoprotein, has been implicated in a wide range of physiologic functions such as lipid transport, tissue repair and remodelling, membrane protection, and promotion of cell interactions.62 Recent studies have also demonstrated that induction of clusterin during the development of atherosclerosis may represent a protective response to the oxidative stress associated with the development of atherosclerosis.63,64 Furthermore, because clusterin is a novel potent inhibitor of complementation-mediated cytolysis, the ability of homocysteine to decrease clusterin gene expression could potentially enhance complement activation at the site of vessel wall damage, which results in increased EC injury and/or dysfunction. PAG, a novel antioxidant protein family member responsive to oxidative stress,65,66 induces cell growth and differentiation by blocking c-Abl tyrosine kinase activity. The ability of homocysteine to decrease PAG expression would not only enhance the cytotoxic effects of oxidants but could suppress EC growth and differentiation.

Although we have identified several gene pathways by which homocysteine could potentially influence EC function and growth, a number of relevant issues remain to be explored. Additional studies are needed to determine if these homocysteine-dependent changes in gene expression in cultured vascular EC are observed in vivo. The fact that GRP78 is induced in the livers of CBS-deficient mice,26 and that the activity of several antioxidant enzymes is altered in rabbits fed a high methionine diet68 supports our findings and suggests that our in vitro studies likely reflect the actions of homocysteine in vivo. Furthermore, it is not known if the ability of homocysteine to cause ER stress directly influences EC growth. Based on the observation that agents known to adversely affect ER function cause inhibition of protein synthesis69 and cell growth,70-72 it is likely that elevated levels of intracellular homocysteine act in a similar fashion. As a result of previous studies demonstrating that homocysteine induces smooth muscle cell proliferation and increases the expression of the cyclin genes,29 it will be of interest to determine if the changes in gene expression observed in smooth muscle cells by homocysteine inversely correlate with some of the genes identified in these studies.

In summary, we have shown that homocysteine-induced ER stress and growth arrest in vascular EC involves changes in gene expression specific for these effects. Furthermore, the ability of homocysteine to decrease the expression of several antioxidant enzymes suggests that homocysteine could indirectly enhance the effects of agents or conditions known to cause oxidative stress. We also show the utility of cDNA microarrays as an initial screening approach for the identification of homocysteine-responsive genes in EC. Based on the high yield of information obtained using an array of fewer than 600 known human genes, a more comprehensive survey of gene expression patterns, using a more complete array of human genes, will not only provide additional important information on the mechanism by which homocysteine promotes EC dysfunction but will also increase our understanding of the gene pathways involved in the pathogenesis of HH.

ACKNOWLEDGMENT

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55. Price BD, Calderwood SK: Gadd45 and Gadd153 messenger RNA levels are increased during hypoxia and after exposure of cells to agents which elevate the levels of the glucose-regulated proteins. Cancer Res 52:3841, 1992


Homocysteine-Induced Endoplasmic Reticulum Stress and Growth Arrest Leads to Specific Changes in Gene Expression in Human Vascular Endothelial Cells

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