Characterization of the stress-inducing effects of homocysteine

P. Andrew OUTINEN*, Sudesh K. SOOD*, Patricia C. Y. LIAW*, Kevin D. SARGE†, Nobuyo MAEDA‡, Jack HIRSH*, José RIBAU*, Thomas J. PODOR*, Jeffrey I. WEITZ* and Richard C. AUSTIN*¹

*Departments of Pathology and Medicine, McMaster University and the Hamilton Civic Hospitals Research Centre, Hamilton, Ontario, Canada L8V 1C3, †Department of Biochemistry, Chandler Medical Center, University of Kentucky, Lexington, KY 40536-0084, U.S.A., and ‡Department of Pathology and Program for Molecular Biology and Genetics, University of North Carolina, Chapel Hill, NC 27599-7525, U.S.A.

The mechanism by which homocysteine causes endothelial cell (EC) injury and/or dysfunction is not fully understood. To examine the stress-inducing effects of homocysteine on ECs, mRNA differential display and cDNA microarrays were used to evaluate changes in gene expression in cultured human umbilical-vein endothelial cells (HUVEC) exposed to homocysteine. Here we show that homocysteine increases the expression of *GRP78* and *GADD153*, stress-response genes induced by agents or conditions that adversely affect the function of the endoplasmic reticulum (ER). Induction of *GRP78* was specific for homocysteine because other thiol-containing amino acids, heat shock or H₂O₂ did not appreciably increase *GRP78* mRNA levels. Homocysteine failed to elicit an oxidative stress response in HUVEC because it had no effect on the expression of heat shock

proteins (HSPs) including *HSP70*, nor did it activate heat shock transcription factor 1. Furthermore homocysteine blocked the H₂O₂-induced expression of *HSP70*. In support of our findings *in vitro*, steady-state mRNA levels of *GRP78*, but not *HSP70*, were elevated in the livers of cystathionine β -synthase-deficient mice with hyperhomocysteinaemia. These studies indicate that the activation of stress response genes by homocysteine involves reductive stress leading to altered ER function and is in contrast with that of most other EC perturbants. The observation that homocysteine also decreases the expression of the antioxidant enzymes glutathione peroxidase and natural killer-enhancing factor B suggests that homocysteine could potentially enhance the cytotoxic effect of agents or conditions known to cause oxidative stress.

INTRODUCTION

Exposure of endothelial cells (ECs) to a variety of oxidative stress conditions causes EC injury and/or dysfunction, which can potentially result in the development of vascular disease and thrombosis [1–4]. Oxidized low-density lipoprotein [5,6], H₂O₂ [7] and ischaemia/reperfusion [8] have previously been shown to cause oxidative damage to ECs through the generation of reactive oxygen species (ROS). In response to these stress conditions, ECs increase their expression and synthesis of heat shock/stress proteins (HSPs), particularly HSP70, which prevent intracellular protein aggregation and subsequent cell damage [9–11]. Oxidative stress conditions also enhance the expression and activity of several antioxidant enzymes such as natural killer-enhancing factor B (NKEFB) [12], glutathione S-transferase [13] and haem oxygenase [14], which serve to limit oxidative damage.

Hyperhomocysteinaemia (HH) is an important independent risk factor for premature atherosclerosis and thrombosis [15–19]. Earlier studies have demonstrated that homocysteine causes EC injury when administered to baboons [20,21] or rats [22], or when added directly to cultured ECs [23–26]. In addition to causing injury, homocysteine can increase the procoagulant activity of cultured ECs by: (1) inducing tissue factor (TF) activity [27], (2) inhibiting protein C activation [28], (3) causing aberrant processing and secretion of thrombomodulin [29], (4) releasing a protease that activates Factor V [30] and (5) reducing the cellular binding sites for tissue plasminogen activator [31]. In addition to its prothrombotic effects, homocysteine can alter the vasoactive properties of cultured ECs by impairing the production of nitric oxide [32], a finding consistent with recent reports indicating that diet-induced HH in monkeys [33] or pigs [34] is associated with

impaired vasomotor regulatory function. Although these studies provide ample evidence that homocysteine causes EC injury and/or dysfunction, the underlying mechanism responsible for the cytotoxic effects of homocysteine is not fully understood.

It has been suggested that the stress-inducing effects of homocysteine on EC function reflect oxidative damage because the highly reactive thiol group of homocysteine is readily oxidized in plasma or culture medium, resulting in the generation of ROS [23–26,35]. Although recent studies indicate that homocysteine induces the steady-state mRNA levels of 78 kDa glucose-regulated protein (GRP78) [36], a member of the 70 kDa HSP family [9,37,38], it is not yet known whether the cellular stress response elicited by homocysteine is similar to those conditions that cause oxidative stress. Here we demonstrate that the stress-inducing effects of homocysteine are fundamentally different from those of other EC perturbants known to cause oxidative damage and might have important implications in the pathophysiology of HH.

MATERIALS AND METHODS

Cell culture and treatment conditions

Human umbilical-vein endothelial cells (HUVEC) were isolated by the treatment of human umbilical veins with collagenase [39] and cultured in EC medium [M199 medium containing 20% (v/v) fetal bovine serum, 20 μ g/ml EC growth factor, 90 μ g/ml pig intestinal heparin, 100 μ g/ml penicillin and 100 μ g/ml streptomycin] in a humidified incubator at 37 °C with air/CO₂ (19:1) [40]. Cells from passages 2–4 were used in these studies. D,L-Homocysteine and CuSO₄ (Sigma, St. Louis, MO, U.S.A.) were prepared in EC medium, sterilized by filtration and added to

Abbreviations used: CBS, cystathionine β -synthase; DTT, dithiothreitol; EC, endothelial cell; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, 78 kDa glucose-regulated protein; HH, hyperhomocysteinaemia; HSE, heat shock element; HSF1, heat shock transcription factor 1; HSP, heat shock protein; HUVEC, human umbilical-vein endothelial cells; NKEFB, natural killer-enhancing factor B; ROS, reactive oxygen species; TF, tissue factor.

¹ To whom correspondence should be addressed (e-mail raustin@thrombosis.hhsr.org).

70 % confluent cell cultures at the desired concentrations. For heat shock treatment, cells were heated in a humidified incubator at 42 °C for the indicated periods. For H₂O₂ treatment, a 30 % (v/v) stock of H₂O₂ (Sigma) was diluted in EC medium to a final concentration of 5 mM immediately before use. Cells were then exposed to 5 mM H₂O₂ for 20 min, washed three times with M199 medium and allowed to recover in EC medium at 37 °C for the indicated periods. After treatment, HUVEC were washed twice with PBS before the isolation of RNA and proteins.

Cystathionine β -synthase (CBS)-deficient mice

Livers were isolated from 10-month-old wild-type, heterozygous and homozygous CBS-deficient mice [41] and washed briefly in ice-cold PBS before storage at -70 °C.

Preparation of total RNA and Northern blot analysis

Total RNA was isolated from cells or tissues by using the RNeasy total RNA kit (Qiagen, Chatsworth, CA, U.S.A.) and resuspended in diethyl pyrocarbonate-treated water. For RNA used in the differential display technique, contaminating DNA was removed by incubation with 2 units of RQ DNase (Promega, Mississauga, ON, Canada) in the presence of 12 units of RNasin (Promega) for 30 min at 37 °C. Quantification and purity of the RNA were assessed by A_{260}/A_{280} absorption, and RNA samples with ratios above 1.6 were stored at -70 °C for further analysis.

Total RNA (10 μ g per lane) was fractionated on 2.2 M formaldehyde/1.2 % (w/v) agarose gels and transferred overnight to Zeta-Probe GT nylon membranes (Bio-Rad, Toronto, ON, Canada) in 10 $\times$ SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate). The RNA was cross-linked to the membrane with a UV cross-linker (PDI Bioscience, Toronto, ON, Canada) before hybridization. Specific probes were generated by labelling the cDNA fragments with [α -³²P]dCTP (specific radioactivity more than 3000 Ci/mmol; NEN, Guelph, ON, Canada) with a randomly primed DNA labelling kit (Boehringer Mannheim, Laval, QC, Canada). After hybridization overnight at 43 °C, the membranes were washed as described by the manufacturer and exposed to Reflections NEF-496 autoradiography film (NEN) at -70 °C for 1–3 days. Changes in the expression of *GRP78* and *HSP70* mRNA transcripts were quantified by densitometric scanning of the blots with the ImageMaster VDS and Analysis Software (Pharmacia Biotech, Mississauga, ON, Canada). To correct for differences in gel loading, integrated optical densities were normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

mRNA differential display

mRNA differential display was performed with RNA image kits (GenHunter, Brookline, MA, U.S.A.) as described by the manufacturer. The cDNA species were amplified by PCR in the presence of [α -³⁵S]dATP (specific radioactivity more than 1000 Ci/mmol; NEN) with a Perkin-Elmer 480 thermal cycler. Parameters for the 40-cycle PCR were as follows: denaturation at 94 °C for 30 s, annealing at 40 °C for 120 s and extension at 72 °C for 30 s. After PCR the radiolabelled cDNA fragments were resolved on 6 % (w/v) sequencing gels. For detection of the cDNA fragments, gels were dried without fixation on Whatman 3M filter paper and then subjected to autoradiography with Kodak XAR-5 film for 1–3 days.

Candidate cDNA fragments showing reproducible induction were excised from the dried gel and the DNA was eluted by boiling the gel slice in water for 10 min. Eluted DNA fragments were reamplified by PCR with the same conditions and primer

sets described above except that the dNTP concentration was 20 μ M and no [α -³⁵S]dATP was added. Amplified cDNA fragments were then purified from 1 % (w/v) agarose gels with the QIAEX gel extraction kit (Qiagen, Chatsworth, CA, U.S.A.) and used as probes on Northern blots.

Cloning and sequencing of cDNA fragments

cDNA fragments that were differentially expressed on Northern blots were subcloned into T-ended pBluescript (KS) and sequenced with a modified T7 polymerase system (Sequenase Version 2; USB, Cleveland, OH, U.S.A.). The cDNA sequences were analysed and compared for similarity with those sequences available in the EMBL and GenBank™ DNA databases.

Analysis of differential gene expression with a human cDNA microarray

Poly(A)⁺ RNA was isolated from total RNA using Oligotex resin (Qiagen). To generate radiolabelled cDNA probes, poly(A)⁺ RNA was reverse-transcribed with Moloney-murine-leukaemia virus in the presence of [α -³²P]dATP. The radiolabelled cDNA probes were purified from unincorporated nucleotides by gel filtration in Chroma Spin-200 columns (Clontech, Palo Alto, CA, U.S.A.) and hybridized overnight at 68 °C to a human cDNA microarray consisting of 588 known human genes, as described by the manufacturer (Clontech). After a series of high-stringency washes [three 20 min washes in 2 $\times$ SSC/1 % (w/v) SDS followed by two 20 min washes in 0.1 $\times$ SSC/0.5 % SDS] at 68 °C, the membranes were analysed by autoradiography as described previously.

Immunoblot analysis

Total cellular proteins were resolved by SDS/PAGE [10 % (w/v) gel] under reducing conditions by the method of Laemmli [42]. Gels were either stained with Coomassie Brilliant Blue or transferred electrophoretically to nitrocellulose (Bio-Rad, Toronto, ON, Canada). The immobilized proteins were then immunostained as described previously [43] with antibodies directed against either GRP78 (StressGen, Vancouver, BC, Canada, or Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or HSP70 (StressGen). After incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies, the membranes were developed with Renaissance chemiluminescence reagent (NEN).

Immunohistochemistry and image analysis

HUVEC grown on gelatin-coated coverslips were fixed in 3.5 % (w/v) paraformaldehyde, permeabilized with PBS containing 0.025 % (v/v) Triton X-100 and then incubated with anti-GRP78 antibodies or with the appropriate preimmune IgG (10 μ g/ml) [44]. To detect primary antibody-binding sites, coverslips were washed and stained with FITC-conjugated goat anti-(mouse IgG) (Gibco/BRL; Mississauga, ON, Canada). After washing in PBS, the coverslips were mounted on glass slides and cells were viewed with an epifluorescence microscope (Laborlux S; Leitz, Wetzlar, Germany) equipped with a barrier filter specific for FITC. Images of fluorescently labelled HUVEC were captured with a charge-coupled device video camera module (Sony, Tokyo, Japan) and image analysis/archival software (Northern Exposure; Empix, Mississauga, ON, Canada).

Gel mobility-shift assay

Gel mobility-shift assays on whole cell extracts were performed with a ³²P-end-labelled heat shock element (HSE) oligonucleotide

as described previously [45]. After gel mobility-shift analysis on native 5% (w/v) polyacrylamide gels, heat shock transcription factor 1 (HSF1)–HSE complexes were detected by autoradiography.

Determination of intracellular levels of homocysteine

Control or homocysteine-treated HUVEC were washed extensively with M199 medium and exposed to cell denaturation buffer [25 mM Tris/HCl (pH 7.4)/100 mM NaCl/0.02% BSA/10 mM EDTA/1.0% (v/v) Triton X-100] for 30 min at 4 °C. After centrifugation of the cell lysates at 4 °C to remove cellular debris, the homocysteine levels in the supernatants were measured by the method described by Fortin and Genest [46], which involves the reduction of disulphide bonds with tri-*n*-butylphosphine and derivatization of the thiol groups with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) followed by separation by HPLC with fluorescence detection.

Statistical analysis

Results are presented as means $\pm$ S.D. Differences in the intracellular levels of homocysteine were tested with a one-way analysis of variance and differences between the control and homocysteine-treated groups were analysed with a Dunnett test. For all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Effect of homocysteine on HUVEC

Exposure of HUVEC to homocysteine (0.1–5.0 mM) for up to 24 h had no effect on overall cell number or viability as determined by Trypan Blue exclusion and ^{51}Cr release assays. As shown in Table 1, HUVEC exposed to 5 mM homocysteine, but not to 1 mM homocysteine, for 2, 4, 8 or 18 h had significantly higher intracellular homocysteine levels than untreated control HUVEC ($P < 0.05$).

Identification of homocysteine-inducible genes in HUVEC

To identify homocysteine-inducible genes, mRNA differential display [47] was used to compare RNA derived from HUVEC cultured in the absence of homocysteine with RNA derived from HUVEC cultured for 18 h in the presence of 1.0 or 5.0 mM homocysteine; 72 primer combinations consisting of three de-

Figure 1 Identification of a homocysteine-inducible gene in HUVEC by mRNA differential display

Total RNA was obtained from HUVEC cultured for 18 h in the absence or presence of 1 or 5 mM homocysteine (hc). The profile of amplified cDNA fragments was reproducible between samples from different experiments and between PCR reactions performed with the same RNA. Two experiments with different primers are shown. **(A)** Band designated HAHAP11-1, which was amplified with primers 5'-AAGCTTCGGGTAA-3' and 5'-AAGCT₁₁A-3', corresponds to a homocysteine-inducible gene. As a positive internal control for homocysteine-induced gene expression, amplification of the 3' end of human TF was performed with primers 5'-AAGCT₁₁A-3' and tf1 (5'-ATGTGTTAATGTC-3'). The band designated tf corresponds to the expected 322 bp amplified TF product in HUVEC treated with homocysteine for 18 h. **(B)** Northern blot analysis of total RNA (10 µg per lane) isolated from HUVEC cultured for 18 h in the absence or presence of 1.0 or 5.0 mM homocysteine with a radiolabelled HAHAP11-1 cDNA probe (top panel). The positions of 18 S and 28 S rRNA are indicated as size markers. The position of a 2.5 kb mRNA transcript (designated HAHAP11-1) induced by homocysteine is indicated by the arrowhead. Hybridization to a *GAPDH* cDNA probe (middle panel) was used to normalize RNA loading. The integrity of the RNA was confirmed by staining the gel with ethidium bromide (bottom panel).

generate anchored oligo(dT) primers (AAGCT₁₁A, AAGCT₁₁C or AAGCT₁₁G) and 24 short arbitrary 13mers (HAP1 to HAP24) were used in these experiments. To confirm differentially expressed cDNA species, and to eliminate false positives, mRNA differential display was done at least twice on RNA isolated from different cultures of HUVEC. As a positive internal control for homocysteine-induced gene expression, homocysteine was shown to increase the expression of human TF mRNA in HUVEC on differential display gels (Figure 1A), a result consistent with previous reports [27].

A total of 40 cDNA fragments were shown to be reproducibly up-regulated in HUVEC cultured in the presence of at least 1 mM

Table 1 Intracellular homocysteine levels in HUVEC treated with exogenous homocysteine

HUVEC cultured to 70% confluency were incubated in the absence or presence of homocysteine for the indicated periods and lysed as described in the Materials and methods section. Intracellular levels of homocysteine were measured from whole cell lysates with HPLC and are presented as means $\pm$ S.D. ($n = 6$ for 0 h control; $n = 3$ for treated samples). * $P < 0.05$ compared with the intracellular concentrations of homocysteine from HUVEC cultured in medium alone (0 h).

Time (h)	Exogenous [homocysteine] (mM) ... 5.0		1.0	
	Intracellular [homocysteine]		Intracellular [homocysteine]	
	(nmol/mg)	(mean fold increase)	(nmol/mg)	(mean fold increase)
0	0.44 $\pm$ 0.22	1.0	0.44 $\pm$ 0.22	1.0
2	2.06 $\pm$ 0.39	4.7*	0.80 $\pm$ 0.04	1.8
4	2.31 $\pm$ 0.19	5.3*	0.74 $\pm$ 0.10	1.7
8	2.42 $\pm$ 0.46	5.5*	0.70 $\pm$ 0.20	1.6
18	1.64 $\pm$ 0.16	3.7*	0.80 $\pm$ 0.08	1.8

Figure 2 Time- and dose-dependent effects of homocysteine on *GRP78* mRNA levels in HUVEC

Total RNA (10 µg per lane), isolated from HUVEC cultured in the presence of 5.0 mM (left panels) or 1.0 mM (right panels) homocysteine for the indicated periods up to 18 h, was analysed for *GRP78* mRNA expression by Northern blot hybridization with a radiolabelled *GRP78* cDNA probe, followed by autoradiography (upper panels). The arrowhead indicates the migration position of *GRP78* mRNA transcripts. Hybridization to a *GAPDH* cDNA probe (lower panels) was used to normalize RNA loading. The results are representative of three separate experiments.

homocysteine. Northern blot analysis revealed that one of these cDNA fragments, designated HAHAP11-1 (Figure 1A), hybridized to a transcript of approx. 2.5 kb, which was increased more than 20-fold after 18 h exposure to 5.0 mM homocysteine (Figure 1B). This transcript was not induced by exposure to 1.0 mM homocysteine for 18 h, a finding consistent with our initial observations on differential display gels. Searching of the GenBank and EMBL databases revealed that the cloned 147 bp HAHAP11-1 cDNA fragment was identical in sequence (excluding the *Hind*III sequence contained within the anchor and arbitrary primers) with the 3' end of the *GRP78* gene. None of the remaining homocysteine-inducible cDNA fragments had any significant sequence similarity to other HSPs (results not shown).

Differential effects of homocysteine on *GRP78* and *HSP70* mRNA levels in HUVEC

Previous studies have shown that homocysteine induces *GRP78* mRNA levels in vascular ECs [36]. For a better characterization of this effect, HUVEC were exposed to increasing concentrations of homocysteine for various periods up to 18 h and steady-state *GRP78* mRNA levels were determined by Northern blot analysis. Exposure to 5 mM homocysteine increased *GRP78* mRNA levels by 2 h, peaking at 8 h (> 20-fold increase) and remained significantly elevated at 18 h (Figure 2). Exposure of HUVEC to 1.0 mM homocysteine also induced expression after 4–8 h (5-fold increase), albeit to a smaller extent than that produced by 5.0 mM homocysteine. Consistent with the differential display findings, *GRP78* mRNA levels returned to control levels by 18 h. The observation that the homocysteine concentration in the culture medium was not significantly lowered by 18 h (results not shown) excludes the possibility that decreases in *GRP78* mRNA levels reflect instability of homocysteine.

In contrast with homocysteine, exposure of HUVEC to 5 mM methionine or cysteine, other thiol-containing amino acids, did not increase *GRP78* mRNA levels at 8 h (Figure 3A) or 18 h (results not shown). Glycine, an amino acid containing a hydrogen atom as its side chain, also failed to induce *GRP78* mRNA levels. However, consistent with previous findings [48],

Figure 3 Effect of various conditions on *GRP78* mRNA levels in HUVEC

(A) Total RNA (10 µg per lane), isolated from HUVEC cultured for 8 h in the absence (control) or presence of 5 mM homocysteine, 5 mM glycine, 5 mM methionine, 5 mM cysteine or 2 mM DTT, was analysed for *GRP78* mRNA induction by Northern blot analysis with a *GRP78* cDNA probe, followed by autoradiography (upper panel). (B) Effect of heat shock, in the absence or presence of either 5 mM homocysteine or 2 mM DTT, on *GRP78* mRNA levels. The migration position of *GRP78* mRNA transcripts is indicated by the arrowhead. Hybridization to a *GAPDH* cDNA probe (lower panel) was used to normalize RNA loading. The results are representative of two separate experiments.

2 mM dithiothreitol (DTT) induced *GRP78* mRNA levels to a similar extent to homocysteine. Heat shock treatment of HUVEC at 42 °C for 3 or 18 h produced a less than 2-fold increase in *GRP78* mRNA levels (Figure 3B), a result consistent with earlier findings [48–51]. However, heat shock failed to block *GRP78* gene induction in the presence of homocysteine or DTT (Figure 3B), suggesting that the cellular factors necessary for *GRP78* gene expression are stable at 42 °C and are distinct from those that signal *HSP70* gene induction.

Homocysteine does not cause a heat-induced stress response in HUVEC

Because *GRP78* is a member of the 70 kDa HSP family, we examined the effect of homocysteine on the steady-state mRNA levels of *HSP70* as well as HSF1-binding activity. There was no change in the mRNA levels of *HSP70* in HUVEC exposed to 5.0 mM homocysteine for up to 18 h (Figure 4A). In contrast, the levels increased markedly in response to heat shock (Figure 4B), a result consistent with previous reports [51–53].

Induction of the cellular stress response is mediated by HSF1, which binds to the HSE present in promoters of *HSP* genes [9,45]. To investigate the effects of homocysteine on the activation of HSF1 and its binding to HSE, HUVEC were cultured in the absence or presence of 5 mM homocysteine for 2, 4 or 18 h at 37 °C, and HSE-binding activity was detected by gel mobility-shift assays (Figure 4C). Exposure to 5 mM homocysteine for 2, 4 or 18 h failed to induce HSE-binding activity (Figure 4C, lanes 3–5), a result consistent with that observed in untreated HUVEC (Figure 4C, lane 1). As expected, heat shock treatment induced the appearance of typical HSF1–HSE complexes (Figure 4C, lane 2). Furthermore, unlike heat shock treatment, homocysteine did not induce hyperphosphorylation of HSF1, on the basis of its migration on SDS/PAGE (results not shown).

Effects of H₂O₂ in the absence or presence of homocysteine, on *GRP78* and *HSP70* mRNA levels in HUVEC

In the presence of Cu²⁺ ions, homocysteine is oxidized in plasma or culture medium to generate H₂O₂, which can cause EC injury

Figure 4 Effect of homocysteine on *HSP70* mRNA levels and on HSF1-HSE binding activity in HUVEC

(A) Total RNA (10 μ g per lane), isolated from HUVEC cultured in the presence of 5.0 mM homocysteine at 37 °C for the indicated periods up to 18 h, was analysed by Northern blot hybridization with a radiolabelled *HSP70* cDNA probe (StressGen), followed by autoradiography (upper panel). The migration position of *HSP70* mRNA transcripts is indicated by the arrowhead. (B) As a positive control for *HSP70* gene induction, HUVEC were heat shocked at 42 °C for 3 h before Northern blot analysis. Hybridization to a *GAPDH* cDNA probe (lower panel) was used to normalize RNA loading. (C) Control HUVEC (lane 1) or HUVEC exposed to either heat shock (hs) at 42 °C for 45 min (lane 2) or to 5 mM homocysteine (hc) for 2, 4 or 18 h (lanes 3–5 respectively) were harvested and whole cell extracts analysed for HSE DNA-binding activity by gel mobility-shift assay with a ^{32} P-end-labelled HSE-containing oligonucleotide probe. Abbreviation: NS, non-specific complexes. The results are representative of two separate experiments.

[23–26]. Like homocysteine alone, the combination of homocysteine and Cu^{2+} ions (Figure 5A) increased *GRP78* mRNA levels without affecting *HSP70* mRNA levels. The observation that H_2O_2 did not appreciably increase *GRP78* mRNA levels (2-fold by 4 and 8 h) compared with H_2O_2 plus homocysteine (10-fold and 20-fold increase by 4 and 8 h respectively) (Figure 5B) indicates that *GRP78* gene induction is due primarily to the effects of homocysteine and not H_2O_2 , and that H_2O_2 does not block *GRP78* gene induction by homocysteine (Figure 5B).

Given that thiol-containing agents block the oxidative stress response [48], the effect of homocysteine on the H_2O_2 -induced expression of *HSP70* in HUVEC was evaluated. Steady-state mRNA levels of *HSP70* increased 10-fold by 2 h, returning to control levels at 4, 8 and 18 h (Figure 5B). Unlike H_2O_2 alone,

Figure 6 Differential effects of homocysteine on *GRP78* and *HSP70* protein levels

Total protein lysates (30 μ g per lane) from HUVEC cultured in the absence or presence of homocysteine (hc) were separated by SDS/PAGE [10% (w/v) gel] and either stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose membranes and immunostained with antibodies against either *GRP78* or *HSP70* (B,C). The migration positions of *GRP78* and *HSP70* are shown by the arrowhead. The results are representative of three separate experiments.

HSP70 levels increased less than 2-fold by 2 h in HUVEC exposed to both H_2O_2 and homocysteine (Figure 5B). Furthermore a 2-fold decrease in the mRNA levels of *HSP70* was observed at 4, 8 and 18 h, compared with control levels.

Effect of homocysteine on *GRP78* and *HSP70* protein levels in HUVEC

To determine the effect of homocysteine on *GRP78* and *HSP70* protein levels, HUVEC were cultured in the absence or presence of 1.0 or 5.0 mM homocysteine and total cell lysates were

Figure 5 Effect of H_2O_2 , in the absence or presence of homocysteine, on *GRP78* and *HSP70* mRNA levels in HUVEC

HUVEC were exposed either to 5.0 mM homocysteine and 4 μ M Cu^{2+} ions (Cu^{++}) (A) or to 5 mM H_2O_2 for 20 min in the absence or presence of 5 mM homocysteine, and allowed to recover at 37 °C for the indicated time periods (B). Total RNA (10 μ g per lane) isolated from control and treated HUVEC was analysed by Northern blot hybridization with radiolabelled cDNA probes to either *GRP78* or *HSP70*, followed by autoradiography. The migration positions of *GRP78* and *HSP70* mRNA transcripts are indicated by the arrowhead. Hybridization to a *GAPDH* cDNA probe (bottom panel) was used to normalize RNA loading. The results are representative of three separate experiments.

Figure 7 Immunolocalization of GRP78 in HUVEC cultured in the absence or presence of homocysteine

HUVEC plated on gelatin-coated glass coverslips were treated in the absence (**A**) or presence (**B**) of 5.0 mM homocysteine for 18 h. After incubation, cells were fixed, permeabilized and incubated with antibodies against GRP78 (**A,B**). Antibody localization was detected with FITC-conjugated goat anti-(mouse IgG) as described (see the Materials and methods section). Parallel experiments with normal mouse IgG (**C**) were performed to assess non-specific immunofluorescence. Magnification $\times 1000$. The results are representative of two separate experiments.

examined by SDS/PAGE followed by immunoblot analysis with antibodies against either GRP78 or HSP70 (Figure 6). Exposure to homocysteine induced the synthesis of a protein band of approx. 78 kDa (Figure 6A), which on immunoblot analysis was identified as GRP78 (Figure 6B). Densitometric scanning revealed that exposure to 1 or 5 mM homocysteine for 18 h increased GRP78 protein levels 2-fold and 5-fold respectively. The finding that homocysteine increased GRP78 protein levels by 8 h (Figure 6C) is consistent with the Northern blot analysis, indicating that *GRP78* mRNA levels are induced before the increase in GRP78 protein levels. In contrast, exposure to homocysteine for periods up to 18 h had no effect on HSP70 protein levels (Figures 6B and 6C).

Homocysteine increases the cellular localization of GRP78 in HUVEC

To examine the effect of homocysteine on the cellular localization of GRP78, HUVEC cultured on coverslips in the absence or presence of 5 mM homocysteine for 18 h were fixed, permeabilized and immunostained for GRP78. In control HUVEC, GRP78 was concentrated in the perinuclear region (Figure 7A),

Figure 8 cDNA microarray analysis of changes in gene expression in HUVEC cultured in the absence or presence of homocysteine

^{32} P-labelled cDNA derived from poly(A)⁺ RNA from control HUVEC (**A**) or HUVEC exposed to 5 mM homocysteine for either 4 h (**B**) or 18 h (**C**) was hybridized to a cDNA microarray. After a series of high-stringency washes, hybridization patterns were analysed by autoradiography. The relative expression levels of specific cDNA species were assessed by comparison with β -actin, 23 kDa highly basic protein (HBP) and ribosomal protein S9 (rS9). Hybridization patterns for *hsp27*, *hsp60*, *hsp70*, *hsp90*, *NKEFB*, glutathione peroxidase (arrowhead) and *gadd153* are indicated.

consistent with its location in the endoplasmic reticulum (ER). In contrast, when HUVEC were treated with 5.0 mM homocysteine for 18 h, both the localization and intensity of GRP78 immunostaining was markedly different (Figure 7B), suggesting an increased synthesis of GRP78 and dilatation of the ER. No specific staining was observed in HUVEC incubated with normal mouse IgG (Figure 7C).

Figure 9 *GRP78* and *HSP70* mRNA levels in livers from wild-type and CBS-deficient mice with HH

Total RNA (10 µg per lane), isolated from the livers of wild-type (+/+), heterozygous (+/−) or homozygous (−/−) CBS-deficient mice was analysed by Northern blot hybridization with cDNA probes to either human *GRP78* or *HSP70*. As a control for RNA loading, the same blots were hybridized with a radiolabelled 18 S rRNA-specific oligonucleotide primer (bottom panel). The results are representative of two separate experiments.

Effect of homocysteine on additional stress response genes

To characterize further the stress-inducing effects of homocysteine on ECs, a human cDNA microarray was screened with radiolabelled cDNA derived from poly(A)⁺ RNA from control HUVEC or from HUVEC exposed to 5 mM homocysteine for 4 or 18 h (Figure 8). Consistent with our Northern blot analysis, mRNA levels of *HSP27*, *HSP60* and *HSP90*. However, mRNA levels of *GADD153*, a gene known to be induced by thiol-induced stress [54], were increased markedly by 4 h, returning to control levels by 18 h. In addition to these stress response genes, mRNA levels of glutathione peroxidase and NKEFB, both of which are members of a family of antioxidant enzymes, were decreased by 4 h, also returning to control levels by 18 h.

Differential expression of *GRP78* and *HSP70* mRNA levels in the livers of CBS-deficient mice with HH

Previous studies have demonstrated liver abnormalities in CBS-deficient mice with HH [38]. To examine whether the steady-state mRNA levels of *GRP78* and/or *HSP70* were altered *in vivo*, total RNA extracted from the livers of wild-type (+/+), heterozygous (+/−) and homozygous (−/−) CBS-deficient mice was subjected to Northern blot analysis (Figure 9). *GRP78* mRNA levels were up-regulated markedly in the livers of homozygous mice compared with both wild-type mice and heterozygotes. In contrast, *HSP70* mRNA levels were unaltered.

DISCUSSION

In this study we describe the stress-inducing effects of homocysteine on vascular ECs and demonstrate that, unlike other EC perturbants, homocysteine causes reductive rather than oxidative stress. This concept is based on several lines of evidence. First, homocysteine increases the expression in ECs of *GRP78* and *GADD153*, stress response genes known to be induced by thiol-

reducing agents that alter ER function. Secondly, homocysteine increases the protein levels of *GRP78* but not *HSP70*. Thirdly, homocysteine has no effect on the expression of HSPs, nor does it activate HSF-1. Fourthly, homocysteine blocks the H₂O₂-induced expression of *HSP70*. Furthermore confirmation of our findings *in vitro* comes from the observation that the mRNA levels of *GRP78*, but not *HSP70*, are increased in the livers of CBS-deficient mice with HH.

The ability of homocysteine to induce the expression and synthesis of *GRP78* is in agreement with the work of other investigators [33] and correlates with an increase in intracellular levels of homocysteine. Because *GRP78* gene expression is induced by stress conditions that cause the accumulation of misfolded or underglycosylated proteins in the lumen of the ER [9,37,38,51], these studies suggest that homocysteine can also adversely affect ER function. Indeed, previous studies have shown that homocysteine can alter the redox potential of the ER, thereby preventing cell-surface expression of thrombomodulin [29] and causing aberrant protein processing and secretion of von Willebrand factor [55]. The finding that *GRP78* mRNA levels eventually decrease, whereas *GRP78* protein levels increase, after exposure to homocysteine is probably related to the levels of free *GRP78* within the lumen of the ER [9,34,35] and/or the induction of cellular factors that influence the metabolism or elimination of intracellular homocysteine. In support of this latter concept, previous studies showed that homocysteine induces the expression of methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase, a bifunctional enzyme involved in homocysteine metabolism [36].

In contrast with homocysteine, other thiol-containing amino acids such as cysteine or methionine, at concentrations up to 5 mM, failed to induce *GRP78* mRNA levels. These findings suggest that (1) HUVEC are much more sensitive to homocysteine than to other thiol-containing amino acids, (2) there is preferential intracellular accumulation of homocysteine relative to methionine or cysteine, and/or (3) HUVEC have the ability to metabolize and/or eliminate methionine and cysteine more rapidly than homocysteine. The observation by Kokame et al. [36] that cysteine concentrations of at least 10 mM are required to induce *GRP78* mRNA levels in HUVEC further implies that ECs are more sensitive to homocysteine than to cysteine.

The ability of homocysteine to induce the expression of *GADD153*, a member of the C/EBP gene family of transcription factors responsive to ER stress [56,57], further suggests that homocysteine adversely affects ER function. On the basis of previous studies demonstrating that DTT induces the expression of both *GRP78* and *GADD153* [54,58], our findings are not surprising. However, the physiological significance of *GADD153* induction by homocysteine remains unclear. Given that homocysteine inhibits EC growth [59], *GADD153* may have a potential role in linking ER stress to alterations in the expression of genes involved in cell growth and proliferation.

The observation that homocysteine has no effect on the expression of HSPs suggests that homocysteine does not induce an oxidative stress response. A survey of the conditions/agents capable of eliciting oxidative stress in ECs reveals that most either generate ROS or decrease cellular thiol function [60,61]. Examples of these include heat shock [7], oxidized low-density lipoprotein [5,6], H₂O₂ [7] and ischaemia/reperfusion [8]. Although others have shown that homocysteine, in the presence of Cu²⁺ ions, can generate ROS [23–26,35], failure to induce an oxidative stress response suggests that homocysteine might mediate the expression of HSPs. Our finding that homocysteine blocks the H₂O₂-induced expression of *HSP70* in HUVEC supports this concept and is in agreement with earlier studies

demonstrating that DTT or 2-mercaptoethanol can inhibit the oxidative stress response [48].

On the basis of our findings, how could homocysteine cause EC injury and/or dysfunction? Initially, increased expression of *GRP78* and *GADD153* is likely to enhance cell survival. Indeed, recent studies indicate that overexpression of *GRP78* in COS [62] or renal epithelial cells [63] protects them from ER stress. However, prolonged exposure to homocysteine could potentially alter the abundance of membrane or secretory proteins. In support of this, Dorner et al. [64] have shown that elevated levels of *GRP78* protein can enhance stable association and decrease the secretion efficiency of proteins that would otherwise transiently associate with *GRP78*. Thus the sensitivity of protein transport to inhibition by homocysteine can vary between secretory proteins and/or indicate a variation in the stable association between *GRP78* and these proteins. The observation that there is a variable interaction of von Willebrand factor, tissue plasminogen activator and factor VIII with *GRP78* further supports a mechanism involving differential protein inhibition within the ER [65]. Thus secretory proteins that are highly sensitive to homocysteine-induced alterations in the intracellular redox potential could potentially impair the processing and secretion of other secretory proteins by acting as catalysts for *GRP78* gene induction. As a result, increased intraluminal concentrations of *GRP78* could selectively influence the balance between procoagulant and anticoagulant proteins in ECs.

The inability of homocysteine to induce the expression of HSPs is important because these proteins are critical for the survival of cells exposed to a variety of physiological stresses. Alterations in the expression of HSPs, particularly *HSP70*, has been widely observed in a number of disease states including oxidant injury, ischaemia, cardiac hypertrophy, fever, metabolic diseases, inflammation, infection and tissue trauma [9–11]. The failure of homocysteine to induce *HSP70* gene expression as well as its ability to inhibit H_2O_2 -mediated *HSP70* gene induction might explain why homocysteine is especially injurious to ECs. Furthermore our finding that homocysteine inhibits the expression of antioxidant enzymes such as glutathione peroxidase and NKEFB suggests that homocysteine might enhance the cytotoxic effects of agents or conditions that elicit oxidative damage. The recent observation that homocysteine limits the bioavailability of endothelium-derived relaxing factor/nitrous oxide through a mechanism involving increased production of ROS coupled with impaired ability to detoxify peroxides by glutathione peroxidase [66] supports this concept.

In summary, unlike other EC perturbants that promote oxidative stress, homocysteine causes reductive stress, which probably reflects ER dysfunction. Furthermore the observation that homocysteine inhibits the expression of antioxidant enzymes suggests that homocysteine might also potentiate the cytotoxic effects of ROS. Further studies are needed to identify the mechanism by which homocysteine causes differential expression and synthesis of these cellular stress proteins.

We thank Dr. Steven Hill and Dr. Matthew McQueen for measuring intracellular homocysteine levels. P.A.O. was a recipient of a Douglas C. Russel Memorial Scholarship and an Ontario Graduate Scholarship; R.C.A. is a Research Scholar of the R. K. Fraser Foundation; J.I.W. is a Career Investigator of the Heart and Stroke Foundation of Ontario. This work was supported by the Heart and Stroke Foundation of Ontario (grant no. NA-3307).

REFERENCES

- Ross, R. (1993) *Nature* (London) **362**, 801–809
- Navab, M., Berliner, J. A., Watson, A. D., Hama, S. Y., Territo, M. C., Lusis, A. J., Shih, D. M., Van Lenten, B. J., Frank, J. S., Demer, L. L. et al. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 831–842
- Navab, M., Fogelman, A. M., Berliner, J. A., Territo, M. C., Demer, L. L., Frank, J. S., Watson, A. D., Edwards, P. A. and Lusis, A. J. (1995) *Am. J. Cardiol.* **76**, 18C–23C
- Luscher, T. F., Tanner, F. C., Tschudi, M. R. and Noll, G. (1993) *Annu. Rev. Med.* **44**, 395–418
- Zhu, W., Roma, P., Pellegatta, F. and Catapano, A. L. (1994) *Biochem. Biophys. Res. Commun.* **200**, 389–394
- Zhu, W., Roma, P., Pirillo, A., Pellegatta, F. and Catapano, A. L. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 1104–1111
- Jornot, L., Mirault, M. E. and Junod, A. F. (1991) *Am. J. Respir. Cell Mol. Biol.* **5**, 265–275
- Yellon, D. M. and Latchman, D. S. (1992) *J. Mol. Cell. Cardiol.* **24**, 113–124
- Morimoto, R. I., Tissieres, A. and Georgopoulos, C. (1994) *The Biology of Heat Shock Proteins and Molecular Chaperones*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Hightower, L. E. (1991) *Cell* **66**, 191–197
- Georgopoulos, C. and Welsh, W. J. (1993) *Annu. Rev. Cell Biol.* **9**, 601–634
- Kim, A. T., Sarafian, T. A. and Shau, H. (1997) *Toxicol. Appl. Pharmacol.* **147**, 135–142
- Pinkus, R., Weiner, L. M. and Daniel, V. (1995) *Biochemistry* **34**, 81–88
- Lautier, D., Luscher, P. and Tyrrell, R. M. (1992) *Carcinogenesis* **13**, 227–232
- Ueland, P. M., Refsum, H. and Brattstrom, L. (1993) in *Atherosclerotic Cardiovascular Disease, Hemostasis and Endothelial Function* (Francis, R. B., ed.), pp. 183–236, Marcel Dekker, New York
- Kang, S.-S., Wong, P. W. K. and Malinow, M. R. (1992) *Annu. Rev. Nutr.* **12**, 279–298
- Selhub, J., Jacques, P. F., Bostom, A. G., D'Agostino, R. B., Wilson, P. W. F., Belanger, A. J., O'Leary, D. H., Wolf, P. A., Schaefer, E. J. and Rosenberg, I. H. (1995) *New Engl. J. Med.* **332**, 286–291
- Mayer, E. L., Jacobsen, D. W. and Robinson, K. (1996) *J. Am. Coll. Cardiol.* **27**, 517–527
- McCully, K. S. (1996) *Nature Med.* **2**, 386–389
- Harker, L. A., Slichter, S. J., Scott, C. R. and Ross, R. (1974) *New Engl. J. Med.* **291**, 537–543
- Harker, L. A., Harlan, J. M. and Ross, R. (1983) *Circ. Res.* **53**, 731–739
- Hladovec, J. (1979) *Blood Vessels* **16**, 202–210
- Wall, R. T., Harlan, J. M., Harker, L. A. and Striker, G. E. (1980) *Thromb. Res.* **18**, 113–121
- Starkebaum, G. and Harlan, J. M. (1986) *J. Clin. Invest.* **77**, 1370–1376
- de Groot, P. G., Willems, C., Boers, G. H. J., Gonsalves, M. D., VanAken, W. G. and Mourik, J. A. (1983) *Eur. J. Clin. Invest.* **13**, 405–410
- Dudman, N. P. B., Hicks, C., Wang, J. and Wilcken, D. E. L. (1991) *Atherosclerosis* **91**, 77–83
- Fryer, R. H., Wilson, B. D., Gubler, D. B., Fitzgerald, L. A. and Rodgers, G. M. (1993) *Arterioscler. Thromb.* **13**, 1327–1333
- Rodgers, G. M. and Conn, M. T. (1990) *Blood* **75**, 895–901
- Lentz, S. R. and Sadler, J. E. (1991) *J. Clin. Invest.* **88**, 1906–1914
- Rodgers, G. M. and Kane, W. H. (1986) *J. Clin. Invest.* **77**, 1909–1916
- Hajjar, K. A. (1993) *J. Clin. Invest.* **91**, 2873–2879
- Stamler, J. S., Osborne, J. A., Jarak, O., Rabbini, L. E., Mullins, M., Singel, S. and Loscalzo, J. (1993) *J. Clin. Invest.* **91**, 308–318
- Lentz, S. R., Sobey, C. G., Piegor, D. J., Bhopatkar, M. Y., Faraci, F. M., Malinow, M. R. and Heistad, D. D. (1996) *J. Clin. Invest.* **98**, 24–29
- Rolland, P. H., Friggi, A., Barlatier, A., Piquet, P., Latrille, V., Faye, M. M., Guillou, J., Charpiot, P., Bodard, H., Ghiringhelli, O. et al. (1995) *Circulation* **91**, 1161–1174
- Loscalzo, J. (1996) *J. Clin. Invest.* **98**, 5–7
- Kokame, K., Kato, H. and Miyata, T. (1996) *J. Biol. Chem.* **271**, 29659–29665
- Pelham, H. R. B. (1986) *Cell* **46**, 959–961
- Lee, A. S. (1987) *Trends Biol. Sci.* **12**, 20–23
- Jaffe, E. A., Nachmann, R. L., Becker, C. G. and Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2754
- Kassis, J., Hirsh, J. and Podor, T. J. (1992) *Blood* **80**, 1758–1764
- Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M. R. and Maeda, N. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1585–1589
- Laemmli, U. K. (1971) *Nature (London)* **227**, 680–685
- Austin, R. C., Howard, P. L., D'Souza, V. N. D., Klamut, H. J. and Ray, P. N. (1995) *Hum. Mol. Genet.* **4**, 1475–1483
- Hill, S. A., Shaughnessy, S. G., Joshua, P., Ribau, J., Austin, R. C. and Podor, T. J. (1996) *Blood* **87**, 5061–5073
- Sarge, K. D., Murphy, S. P. and Morimoto, R. I. (1993) *Mol. Cell. Biol.* **13**, 1392–1407
- Fortin, L. and Genest, J. (1995) *Clin. Biochem.* **28**, 155–162
- Liang, P. and Pardee, A. B. (1992) *Science* **257**, 967–971

-
- 48 Huang, L. E., Zhang, H., Bae, S. W. and Liu, A.Y-C. (1994) *J. Biol. Chem.* **48**, 30718–30725
- 49 Braakman, I., Helenius, J. and Helenius, A. (1992) *Nature (London)* **356**, 260–262
- 50 Graven, K. K., Zimmerman, L. H., Dickson, E. W., Weinhouse, G. L. and Farber, H. W. (1993) *J. Cell. Physiol.* **157**, 544–554
- 51 Watowich, S. S. and Morimoto, R. (1988) *Mol. Cell. Biol.* **8**, 393–405
- 52 Jornot, L., Mirault, M. E. and Junod, A. F. (1991) *Am. J. Respir. Cell Mol. Biol.* **5**, 265–275
- 53 Lu, D., Maulik, N., Moraru, I. I., Kruetzer, D. L. and Das, D. K. (1993) *Am. J. Physiol.* **264**, C715–C722
- 54 Halleck, M. M., Holbrook, N. J., Skinner, J., Liu, H. and Stevens, J. L. (1997) *Cell Stress Chaperones* **2**, 31–40
- 55 Lentz, S. R. and Sadler, J. E. (1993) *Blood* **81**, 683–689
- 56 Chen, Q., Yu, K., Holbrook, N. J. and Stevens, J. L. (1992) *J. Biol. Chem.* **267**, 8207–8212
- 57 Wang, X.-Z., Lawson, B., Brewer, J. W., Zinszner, H., Sanjay, A., Mi, L.-J., Boorstein, R., Kreibich, G., Hendershot, L. M. and Ron, D. (1996) *Mol. Cell. Biol.* **16**, 4273–4280
- 58 Whelan, S. A. and Hightower, L. E. (1985) *J. Cell Physiol.* **125**, 251–258
- 59 Tsai, J.-C., Perrella, M. A., Yoshizumi, M., Hsieh, C.-M., Haber, E., Schlegel, R. and Lee, M.-E. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6369–6373
- 60 Loven, L. (1988) *Med. Hypoth.* **26**, 39–50
- 61 Nover, L. (1984) *Heat Shock Responses of Eukaryotic Cells*, Springer-Verlag, Berlin
- 62 Morris, J. A., Dorner, A. J., Edwards, C. A., Hendershot, L. M. and Kaufman, R. J. (1997) *J. Biol. Chem.* **272**, 4327–4334
- 63 Liu, H., Bowes, R. C., van de Water, B., Silience, C., Nagelkerke, J. F. and Stevens, J. L. (1997) *J. Biol. Chem.* **272**, 21751–21759
- 64 Dorner, A. J., Wasley, L. C. and Kaufman, R. J. (1992) *EMBO J.* **11**, 1563–1571
- 65 Dorner, A. J., Wasley, L. C. and Kaufman, R. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7429–7432
- 66 Upchurch, G. R., Welch, G. N., Fabian, A. J., Freedman, J. E., Johnson, J. L., Keaney, J. F. and Loscalzo, J. (1997) *J. Biol. Chem.* **272**, 17012–17017
-