Homocysteine-dependent Alterations in Mitochondrial Gene Expression, Function and Structure

HOMOCYSТЕINE AND H2O2 ACT SYNERGISTICALLY TO ENHANCE MITOCHONDRIAL DAMAGE*

(Received for publication, June 1, 1998, and in revised form, August 14, 1998)

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Mitochondrial abnormalities have been identified in hepatocytes of patients with hyperhomocysteinemia and in endothelial cells from the aortas of rats with diet-induced hyperhomocysteinemia. However, the mechanism by which homocysteine affects mitochondria is unknown. In this report, homocysteine-induced expression of the mitochondrial electron transport chain gene, cytochrome c oxidase III/ATPase 6,8 (CO3/ATPase 6,8), was identified in a human megakaryocytic cell line DAMI using mRNA differential display. Steady-state mRNA levels of CO3/ATPase 6,8, as well as other mitochondrial transcripts, were increased in DAMI cells by homocysteine in a concentration- and time-dependent manner. Despite an increase in mitochondrial RNA levels and changes in mitochondrial ultrastructure, no effect on either cell growth or mitochondrial respiration rates was observed in DAMI cells exposed to homocysteine at concentrations up to 1 mM. In contrast, 1 mM homocysteine in the presence of Cu2⁺, which is known to generate H2O2, significantly decreased mitochondrial RNA levels, caused gross morphological changes in mitochondrial ultrastructure, and inhibited both cell growth and mitochondrial respiration rates. However, precursors of cellular glutathione and preexposure to heat shock blocked the decrease in mitochondrial RNA levels caused by homocysteine and Cu2⁺. The observations that (i) homocysteine and H2O2, but not H2O2 alone, caused a decrease in mitochondrial RNA levels, (ii) intracellular levels of H2O2 were significantly increased in the presence of homocysteine and Cu2⁺, and (iii) catalase, but not free radical scavengers, prevented a decrease in mitochondrial RNA levels, provide evidence that homocysteine and H2O2 act synergistically to cause mitochondrial damage. Furthermore, our findings suggest that intracellular glutathione and heat shock proteins play a role in protecting mitochondria against the adverse effects elicited by homocysteine and H2O2.

Homocysteine is a thiol-containing amino acid that is formed when methionine is converted to cysteine. Once synthesized, homocysteine may either be metabolized to cysteine by the transsulfuration pathway or remethylated to methionine (1–5). Deficiencies of the enzymes involved in these pathways (i.e., cystathionine β-synthase and 5,10-methylenetetrahydrofolate reductase) and/or cofactors necessary for the metabolism of homocysteine (i.e., vitamin B6, vitamin B12, or folate) can cause aberrant intracellular processing of homocysteine, leading to hyperhomocysteinemia (HH).

The most common hereditary causes of HH result from mutations in the genes encoding cystathionine β-synthase or 5,10-methylenetetrahydrofolate reductase. Patients with severe HH due to cystathionine β-synthase or 5,10-methylenetetrahydrofolate reductase deficiency exhibit a wide range of clinical manifestations, including mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities, and a fatty liver (6). The major cause of death in these patients is premature vascular and thrombotic disease (6–8), and recent evidence suggests that patients with mild HH also have an increased risk of cardiovascular disease (1–5, 9, 10).

Experimental evidence indicates that homocysteine causes cell injury when administered to animals (11–13) or when added directly to cultured mammalian cells (14–17). It has been suggested that homocysteine-induced cell injury involves oxidative damage because homocysteine is readily oxidized in plasma to form reactive oxygen species, such as H2O2, and cell injury caused by homocysteine is blocked by catalase (14, 15). More recently, we demonstrated that, unlike their response to other oxidative stress conditions, exposure of cultured human vascular endothelial cells (ECs) to homocysteine fails to elicit an oxidative stress response (18). Instead, homocysteine causes reductive stress with decreased expression of several antioxidant enzymes and subsequent endoplasmic reticulum dysfunction. Given that homocysteine decreases intracellular glutathione peroxidase activity (19), these studies raise the possibility that homocysteine may enhance the cytotoxic effect of agents or conditions known to generate reactive oxygen species. The purpose of this study was to test this possibility.

In this investigation, we demonstrate that homocysteine alters mitochondrial gene expression, structure, and function and that severe mitochondrial damage occurs in the presence of homocysteine and H2O2. These studies support a mechanism whereby homocysteine and H2O2 act synergistically to cause cellular injury.

* The abbreviations used are: CO3/ATPase 6,8, cytochrome c oxidase III + ATPase 6,8; ND5, NADH-coenzyme Q oxidoreductase subunit 5; HH, hyperhomocysteinemia; EC, endothelial cell; HSP60, 60-kDa heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; NAC, N-acetyl-L-cysteine; PBS, phosphate-buffered saline; DCF, dichlorodihydrofluorescein; GSH, glutathione.
Homocysteine-induced Effects on Mitochondria

mitochondrial damage, thereby promoting cell injury and/or dysfunction.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment Conditions**—The human megakaryocytic cell line DAMI was obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2. Homocysteine, homoserine, methionine, cysteine, S-adenosyl-L-methionine (SAM), S-adenosyl-L-homocysteine (SAH), N-acetyl-L-cysteine (NAC), dl-dithiothreitol, cupric sulfate (CuSO4), and catalase (Sigma) were prepared in RPMI 1640 medium, which was filtered and added to cell cultures at the desired concentrations. For heat shock experiments, DAMI cells were heated for 2 h at 42 °C in RPMI medium containing 5% fetal bovine serum. After exposure to heat, cells were allowed to recover at 37 °C for 6 or 18 h and then treated with homocysteine, in the absence or presence of Cu2+. Unless otherwise stated, DAMI cells were used at a concentration of 1 × 106 cells/ml. After incubation for the appropriate time period, cell number and viability were assessed by counting cells in a hemacytometer.

**RNA Isolation and Purification**—All solutions were prepared with water that had been treated with 0.1% diethyl pyrocarbonate (Sigma) and autoclaved. Following treatment, DAMI cells were washed with PBS and pelleted by centrifugation. Total RNA was isolated from cells using the RNaseasy total RNA kit as described by the manufacturer (Qiagen, Chatsworth, CA). For mRNA differential display experiments, contaminating DNA was removed by incubation of the RNA with 2 units of RQ DNase (Promega, Mississauga, Ontario, Canada) and 12 units of RNasin (Promega) for 30 min at 37 °C. After the addition of diethyl pyrocarbonate-treated water to 100 μl samples, were mixed with an equal volume of phenol/chloroform (3:1) and centrifuged, and the aqueous phase was transferred to an RNase-free, 1.7-ml centrifuge tube. After the addition of 10 μl of 3 M sodium acetate and 300 μl of absolute ethanol, the RNA was precipitated overnight at −70 °C. RNA was collected by centrifugation at 14,000 × g for 10 min at 4 °C, washed briefly with 85% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. RNA samples with A260/A280 ratios above 1.6 were stored at −70 °C for further analysis.

**mRNA Differential Display—**mRNA differential display was performed using the RNA map kits (GenHunter, Brookline, MA) as described previously (18). Candidate cDNA fragments excised from the display gel were amplified by PCR, purified from 1% agarose gels, and used as probes on Northern blots.

**Cloning and Sequencing of cDNA Fragments—**Amplified cDNA fragments were subcloned into T-ended pBluescript II (KS) (Stratagene), used as probes on Northern blots. Display gel were amplified by PCR, purified from 1% agarose gels, and used as probes on Northern blots.

**Northern Blot Analysis—**Total RNA was fractionated on 2.2% formaldehyde/1.2% agarose gels and transferred overnight onto Zeta-Probe GT nylon membranes (Bio-Rad) in 10× SSC. The RNA was cross-linked to the membrane using a Stratalinker UV cross-linker (PDI Bioscience, OR), or HSP60 (StressGen). Protein concentrations were determined using the Bio-Rad DC protein assay kit with bovine IgG as the standard.

**Transmission Electron Microscopy—**Control or treated DAMI cells were pelleted, washed with PBS, and fixed for 2 h in 2% glutaraldehyde and 1% OsO4 in PBS. Cells were then washed, dehydrated in a graded alcohol series, and embedded in Spurr’s resin. Thin sections (70 nm) were stained with uranyl acetate and examined using a Jeol 100B electron microscope. A minimum of 10 micrographs were evaluated for each experimental condition.

**Mitochondrial Respiration Rates—**To measure mitochondrial respiration rates in control or homocysteine-treated DAMI cells, steady-state oxygen consumption of whole cells permeabilized with digitonin (0.005%) was quantified at 37 °C using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). After treatment, cells were pelleted by centrifugation and resuspended in respiration buffer (0.25 mM succrose, 0.01 mM MgCl2, 0.02 mM HEPES, 0.02 mM KH2PO4, pH 7.1) at a concentration of 1 × 106 cells/ml. To each 100-μl aliquot of cell suspension, 1 μl of digitonin diluted in 0.5% dimethyl sulfoxide, and 610 μl of the suspension was then loaded into the microchamber. Trypan blue uptake was used to confirm plasma membrane permeabilization. After measuring baseline respiration rates, 5 mM succinate and 1 mM adenosine diphosphate were added to maximally stimulate mitochondrial respiration rates. Respiration rates were measured in the absence or presence of 1 μM oligomycin, a specific inhibitor of mitochondrial oxidative phosphorylation, so that the increase attributable to mitochondria could be determined. Respiration rates were calculated as percentage of oxygen consumed per min per 1 × 106 cells, and air-saturated buffer was considered as 100%.

**Detection of Intracellular H2O2 in Dami Cells—**Intracellular levels of H2O2 were determined by flow cytometry using the nonfluorescent probe 2′,7′-dichlorofluorescin (DCF) diacetate (Molecular Probes, Eugene, OR). Removal of the acetate groups by intracellular esterases results in the release of dichlorofluorescin, which upon exposure to hydroperoxides, such as H2O2 and lipid peroxides, is hydrolyzed to the fluorescent probe dichlorofluorescin (21). DAMI cells (5 × 106 cells/ml) pulsed for 60 min in medium containing 5% fetal bovine serum and 100 μM DCF-diacetate (10 μM stock solution freshly prepared in dimethyl sulfoxide) were vortexed and resuspended in fresh media without DCF-diacetate. Cells were then treated for 60 min in medium containing either Cu2+, homocysteine, homocysteine and Cu2+, or H2O2. A minimum of 5 × 106 cells from each treatment were analyzed to obtain a distribution of population fluorescence at 525 nm for emission and 488 nm for excitation using an EPICS PROFILE II flow cytometer (Coulter, Burlington, Ontario, Canada). Data represent a comparison of treated versus control samples in which control means were set to 100% in each individual test.

**Determination of Intracellular Glutathione Levels—**Intracellular glutathione (GSH) was measured using the BIOXYTECH GSH-400 colorimetric assay as described by the manufacturer (OXIS International, Portland, OR). Briefly, DAMI cells (1 × 106 cells/ml) were incubated for 4 h in control medium or medium containing either homocysteine or NAC. After washing in Hank’s balanced saline solution, cells were snap-frozen on dry ice, homogenized in ice-cold 2.5% (w/v) metaphosphoric acid (Sigma), and centrifuged at 4 °C to remove any insoluble material. Samples were measured at a final absorbance of 400 nm in a LKB Ultraspec II spectrophotometer (Amersham Pharmacia Biotech), and intracellular GSH levels were determined from a standard curve using reduced GSH (Sigma).

**Determination of Mitochondrial Mass—**Mitochondrial mass was measured by flow cytometry using nonyl acridine orange (Molecular Probes), a fluorescent dye capable of binding mitochondrial cardiolipin in an energy-independent manner. DAMI cells were seeded into 24-well plates at a concentration of 5 × 105 cells/ml in RPMI medium containing 5% fetal bovine serum. After a 4-h adjustment period in fresh medium, cells were treated for 18 h in medium containing either Cu2+, homocysteine, or homocysteine and Cu2+. Nonyl acridine orange was then added to a final concentration of 0.1 μM for 45 min, and the fluorescence distribution of each treated cell population was evaluated by flow cytometry.

**Statistical Analysis—**Data are presented as the means ± S.E. Significance of differences between control and treated groups was deter-

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In this document, we describe the effects of homocysteine on mitochondrial function in DAMI cells. Homocysteine was used as a probe to identify changes in gene expression and mitochondrial respiration rates. The effects were monitored using Northern blot analysis, transmission electron microscopy, and flow cytometry. The data indicate that homocysteine induces mitochondrial dysfunction, which may be related to increased intracellular levels of H2O2 and glutathione. These findings suggest potential mechanisms by which homocysteine may contribute to cardiovascular disease.
**TABLE I**

| Treatment                  | Live cells (%) | S.E. from three independent experiments |
|----------------------------|----------------|----------------------------------------|
| Control                    | 93 ± 0.6       |                                        |
| Control + 4 μM Cu2⁺        | 87 ± 2.6       |                                        |
| 1 μM homocysteine          | 93 ± 1.0       |                                        |
| 5 μM homocysteine          | 87 ± 1.9       |                                        |
| 1 μM homocysteine + 4 μM Cu2⁺ | 90 ± 1.8    |                                        |
| 5 μM homocysteine + 4 μM Cu2⁺ | 54 ± 4.4      |                                        |
| 1 μM homocysteine + 4 μM Cu2⁺ + catalase | 89 ± 0.6 | (2500 units/ml)                        |
| 5 μM homocysteine + 4 μM Cu2⁺ + catalase | 80 ± 1.7      | (2500 units/ml)                        |
| Catalase (2500 units/ml)   | 93 ± 1.5       |                                        |

Effect of homocysteine, in the absence or presence of Cu²⁺, on DAMI cell viability

DAMI cells (1 x 10⁶ cells/ml) were incubated in control or test medium supplemented with 5% fetal bovine serum at 37°C for 18 h. Cell viability was assessed by counting cells on a hemocytometer after suspension in PBS containing 0.5% trypan blue stain. The data represent the means ± S.E. from three independent experiments.

**RESULTS**

Effect of Homocysteine ± Cu²⁺ on Cell Viability and Growth—Earlier studies have shown that the presence of Cu²⁺, homocysteine is readily oxidized in plasma or culture medium to generate H₂O₂, which can cause cell lysis (14, 15). As illustrated in Table I, exposure of DAMI cells (1 x 10⁶ cells/ml) to 1 μM homocysteine for up to 18 h, in the absence or presence of Cu²⁺, had no effect on cell viability. However, viability was dependent on initial cell density because DAMI cells seeded at <1 x 10⁵ cells/ml were lysed when exposed to homocysteine and Cu²⁺ for 18 h. In contrast, neither Cu²⁺ nor catalase alone had any significant effect on cell viability.

To examine the effect on cell growth, DAMI cells (1 x 10⁵ cells/ml) were exposed to varying concentrations of homocysteine, in the absence or presence of Cu²⁺, for periods up to 7 days (Fig. 1). Although 1 μM homocysteine had no significant effect on growth rates, 5 μM homocysteine significantly reduced growth (p < 0.01) beyond 2 days. Unlike 1 μM homocysteine alone, exposure to 1 μM homocysteine and Cu²⁺ completely blocked cell growth and led to cell lysis by 48 h. In contrast, Cu²⁺ alone had no significant effect on cell growth rates (data not shown).

Identification of Cytochrome c Oxidase/ATPase 6,8 as a Homocysteine-inducible Gene—To identify changes in gene expression induced by homocysteine, we compared cDNA differential display patterns from DAMI cells exposed to 1 μM homocysteine for 18 h with those from cells cultured in the absence of homocysteine. After screening through 48 primer combinations, a total of 10 differentially expressed cDNA fragments were identified on sequencing gels. Among these, a cDNA fragment amplified with T₁₂MG and AP-1 primers, designated dhc-1 (Fig. 2A), was shown to be reproducibly up-regulated in DAMI cells exposed to homocysteine. Initial Northern blot hybridization experiments using RNA from control or homocysteine-treated DAMI cells indicated that the dhc-1 cDNA fragment hybridized to a human cDNA probe of ~1.8 kb in size, which corresponded to the primary and mature transcripts of COXIII/ATPase 6,8 (CoxIII/ATPase 6,8), a mitochondrial electron transport chain transcript encoded by mitochondrial DNA.

To confirm that expression of COXIII/ATPase 6,8 in DAMI cells was induced by homocysteine, total RNA from cells grown in the absence or presence of increasing concentrations of homocysteine was analyzed by Northern blot hybridization, using the 16-1 cDNA insert as a probe. Of significance, 16-1 was shown to hybridize to two major transcripts of approximately 1.8 and 0.9 kb, respectively (Fig. 2C), which correspond to the primary and mature transcripts of COXIII/ATPase 6,8 (CoxIII/ATPase 6,8), respectively. Exposure of DAMI cells to increasing concentrations of homocysteine for 18 h caused a dose-dependent increase in the steady-state levels of both transcripts (Fig. 2C). Even exposure to as little as 0.1 μM homocysteine for 18 h increased both the primary and mature COXIII/ATPase 6,8 mRNA levels by 2- and 3-fold, respectively. In addition to homocysteine, other thiol-containing agents, such as dithiothreitol, NAC, and cysteine were shown to induce COXIII/ATPase 6,8 mRNA levels (data not shown). However, this effect was not solely dependent on the thiol group because mRNA levels induced by these agents were lower than those observed for homocysteine.

To determine whether homocysteine increases the expression of other mitochondrial genes, total RNA from control and homocysteine-treated DAMI cells was subjected to Northern blot analysis using the following mitochondrial probes: NDS (component of the electron transport chain), 12S rRNA, 16S rRNA, and HSP60 (nuclear-encoded mitochondrial chaperone). Fig. 2D shows that the steady-state levels of these transcripts also are up-regulated by homocysteine. In contrast, there was no change in the steady-state mRNA levels of GAPDH. Although HSP60 was induced, HSP70 mRNA levels remained unchanged (data not shown), suggesting that induction of HSP60 is not due oxidative stress, a finding consistent with
previous studies (18). Despite an increase in mitochondrial RNA levels upon exposure to homocysteine for 18 h, Western blot analysis using antibodies against cytochrome C oxidase subunits I, II, IV, Vb, or HSP60 indicated no significant increase in mitochondrial protein levels at 18 h (Fig. 3A).

Homocysteine and Cu²⁺ Decrease Mitochondrial RNA and Protein Levels—To determine whether altered mitochondrial gene expression induced by homocysteine involves reactive oxygen species generated by Cu²⁺-catalyzed oxidation of homocysteine, Northern blot analysis was performed on total RNA isolated from DAMI cells exposed to 1 mM homocysteine, in the absence or presence of 1 mM Cu²⁺, for up to 18 h (Fig. 4). As expected, exposure of DAMI cells to 1 mM homocysteine alone caused a time-dependent increase in the mRNA levels of both CO3/ATPase 6,8, and HSP60 (Fig. 4A). In contrast, the steady-state mRNA levels of CO3/ATPase 6,8 and HSP60 decreased in a time-dependent manner when DAMI cells were exposed to both 1 mM homocysteine and Cu²⁺ (Fig. 4B). The absence of smearing of the bands suggests that the decrease in the mRNA levels is likely due to a reduced transcription rate and not degradation. By 18 h, mRNA levels of CO3/ATPase 6,8 and HSP60 were <10% of control levels. However, this effect was completely blocked by the addition of catalase (Fig. 4C). Additional mitochondrial transcripts, including ND5, 12S, and 16S rRNAs, also decreased to a similar extent when DAMI cells were exposed to both 1 mM homocysteine and Cu²⁺; however, GAPDH levels were unchanged (data not shown). Exposure of DAMI cells to either Cu²⁺ or catalase alone had no effect on mitochondrial gene expression. Mitochondrial protein levels were also decreased by 18 h in the presence of at least 1 mM homocysteine and Cu²⁺ (Fig. 3B); however, the decrease was not as dramatic as that observed with RNA levels.

Having shown that homocysteine and Cu²⁺ decrease mitochondrial mRNA levels, we next determined the minimal concentrations of homocysteine and Cu²⁺ necessary to cause this effect. In the presence of 4 μM Cu²⁺, concentrations of homocysteine less than 1 mM did not significantly decrease CO3/ATPase 6,8 mRNA levels (Fig. 5A). Furthermore, concentrations of at least 1 μM Cu²⁺ together with 1 mM homocysteine are needed to decrease mRNA levels to <10% of control levels (Fig. 5A).

In addition to homocysteine, other structurally related molecules were examined for their ability to decrease CO3/ATPase 6,8 mRNA levels in the presence of Cu²⁺ (Fig. 5B). Cysteine, which like homocysteine contains a free thiol group, also causes a decrease in CO3/ATPase 6,8 mRNA levels, albeit to a lesser extent than equimolar concentrations of homocysteine. In contrast, methionine, SAH, SAM, cystathionine, or homoserine had little or no effect on mRNA levels (Fig. 5B) or DAMI cell viability, even in the presence of Cu²⁺ (Table II).

Homocysteine and H₂O₂ Act Synergistically to Decrease Mitochondrial RNA—To determine whether homocysteine and H₂O₂ act synergistically to decrease steady-state mRNA levels of CO3/ATPase 6,8, DAMI cells were exposed to homocysteine in the presence or absence of exogenous H₂O₂. Cells exposed to 0.1 to 1.0 mM H₂O₂ for 18 h showed no change in CO3/ATPase 6,8 mRNA levels (Fig. 6A). In contrast, when cells were exposed to 1 mM homocysteine together with H₂O₂ in concentrations ranging from 0.1 to 1.0 mM, there was a concentration-dependent reduction in steady-state mRNA levels of CO3/ATPase 6,8 (Fig. 6B), an effect prevented by the addition of catalase (Fig. 6C).

Effect of Homocysteine ± Cu²⁺ on Mitochondrial Respiration Rates—Mitochondrial respiration rates were measured to determine whether homocysteine, in the absence or presence of Cu²⁺, altered mitochondrial function (Fig. 7). Although exposure to 1 mM homocysteine for 18 or 36 h had no effect on mitochondrial respiration rates, cells exposed to 5.0 mM homocysteine for 36 h showed a significant decrease (p < 0.01), when compared with control cells or cells exposed to 1 mM homocysteine.
homocysteine (Fig. 7B). When cells were exposed to 1 mM homocysteine together with Cu²⁺, respiration rates were virtually undetectable at 18 h. This effect likely involves depolarization of the mitochondria because the decrease in respiration rates did not correlate with a significant decrease in mitochondrial protein levels (Fig. 3B).

Ultrastructural Changes in Mitochondria from DAMI Cells Exposed to Homocysteine ± Cu²⁺—Based on our observation that homocysteine influences mitochondrial gene expression and function, we examined mitochondrial ultrastructure in DAMI cells exposed to 1 mM homocysteine for 18 h, in the presence or absence of Cu²⁺ (Fig. 8). Compared with controls, cells exposed to homocysteine contained a subpopulation of enlarged mitochondria with fractured cristae (Fig. 8, compare A and B). Mitochondria also tended to be clustered toward the cell center. In addition, large intracytoplasmic vesicles were observed in DAMI cells exposed to homocysteine and Cu²⁺ for 18 h. Control for RNA loading and integrity was assessed by ethidium bromide staining of the 28S and 18S rRNA bands (lower panels).
DCF fluorescence increased approximately 5-fold when DAMI cells exposed to 5 mM homocysteine and glutathione (23, 24), to cells exposed to 5 mM homocysteine and Cu2+. The data represent the means ± S.E. from three independent experiments.

| Treatment | Live cells % |
|-----------|-------------|
| Control   | 82 ± 0.3    |
| 1 mM homocysteine | 77 ± 1.6   |
| 1 mM homocysteine + 4 mM Cu2+ | 74 ± 0.1 |
| 1 mM cysteine | 78 ± 0.9   |
| 1 mM cysteine + 4 mM Cu2+ | 78 ± 0.8  |
| 1 mM methionine | 74 ± 1.5  |
| 1 mM methionine + 4 mM Cu2+ | 83 ± 0.2  |
| 1 mM homoserine | 79 ± 1.4   |
| 1 mM homoserine + 4 mM Cu2+ | 79 ± 3.6  |
| 1 mM SAM | 82 ± 1.2    |
| 1 mM SAM + 4 mM Cu2+ | 75 ± 1.1   |
| 1 mM SAH | 69 ± 1.3    |
| 1 mM SAH + 4 mM Cu2+ | 71 ± 2.0   |
| 1 mM cystathionine | 81 ± 0.9  |
| 1 mM cystathionine + 4 mM Cu2+ | 72 ± 3.4  |

Homocysteine ≥ Cu2+ Increase Intracellular Levels of H2O2 in DAMI Cells—To determine whether homocysteine, in the absence or presence of Cu2+, increases intracellular levels of H2O2, we measured DCF fluorescence using flow cytometry (21). As shown in Fig. 9, incubation of DAMI cells with 1 or 5 mM homocysteine did not significantly increase DCF fluorescence above that found in control or Cu2+-treated cells. In contrast, DCF fluorescence was increased 2–3-fold (p < 0.01) in cells exposed to homocysteine and Cu2+. As a positive control, DCF fluorescence increased approximately 5-fold when DAMI cells were exposed to 1 mM H2O2.

Precursors of Cellular Glutathione, but Not Free Radical Scavengers, Block the Decrease in Mitochondrial RNA Levels—We determined whether precursors of glutathione or free radical scavengers blocked the adverse effects of homocysteine and Cu2+ on mitochondria by examining CO3/ATPase 6,8 mRNA levels. High concentrations of homocysteine can potentially enhance cellular glutathione levels and scavenger H2O2 (15). Unlike 1 or 5 mM homocysteine in the presence of Cu2+, 10 mM homocysteine together with Cu2+ blocked the decrease in CO3/ATPase 6,8 mRNA levels (Fig. 10A). Similarly, the addition of 5 mM NAC, a general antioxidant and precursor of glutathione (23, 24), to cells exposed to 5 mM homocysteine and Cu2+, prevented the decrease in mRNA levels. To better define this protective effect, intracellular levels of GSH were measured from DAMI cells exposed to either homocysteine or NAC. As shown in Table III, DAMI cells exposed to 1 or 10 mM homocysteine showed increases in intracellular GSH of 50 and 113%, respectively, compared with control cells, a result consistent with previous studies using bovine aortic endothelial cells (19). Cells exposed to NAC also showed similar increases in GSH levels. These findings suggest that the protective effect of high levels of homocysteine or NAC result, in part, from an increase in intracellular GSH.

Unlike the protective effects of high concentrations of homocysteine or NAC, free radical scavengers such as mannitol, dimethyl sulfoxide, and superoxide dismutase failed to block the decrease in the mRNA levels of CO3/ATPase 6,8 caused by homocysteine and Cu2+ (Fig. 10B). In addition, superoxide dismutase in the presence of homocysteine alone caused a reduction in mRNA levels. Taken together, these findings suggest that H2O2, but not superoxide anion or hydroxyl radicals, acts preferentially with homocysteine to enhance mitochondrial damage.

Decrease in Mitochondrial RNA Levels by Homocysteine and Cu2+ Is Blocked by Preexposure of DAMI Cells to Heat Shock—Previous studies have demonstrated that heat shock pretreatment of U937 cells prevents H2O2-induced oxidative injury to mitochondria (25). To examine whether heat shock pretreatment also protects mitochondria from the effects of homocysteine and Cu2+, DAMI cells preexposed to heat shock (followed by a 6- or 18-h recovery period), were treated with homocysteine and Cu2+. As shown in Fig. 11, heat shock pretreatment prevented the decrease in the mRNA levels of both CO3/ATPase 6,8 and HSP60 (Fig. 11, compare lanes 1 and 2 with lanes 4–7). Preexposure to heat shock alone did not alter mitochondrial RNA levels (compare lanes 1 and 3).

DISCUSSION

Previous studies have shown mitochondrial abnormalities in ECs from the aortas of hypertensive rats with diet-induced HH (26) and in hepatocytes of patients with HH (4, 27). Although these studies suggest that homocysteine adversely affects mitochondria, the mechanism of action is unknown. In this study, we demonstrate that homocysteine alters mitochondrial gene expression, function, and structure and provide evidence that homocysteine and H2O2 act synergistically to enhance mitochondrial damage. Furthermore, our findings suggest that intracellular glutathione levels and heat shock proteins play an important role in protecting mitochondria from the damaging effects of homocysteine and H2O2.

Although 1 mM homocysteine had no effect on cell growth or mitochondrial respiration rates, it induced changes in mitochondrial ultrastructure and increased mitochondrial RNA levels. Similar increases in mitochondrial RNA levels have also been observed in the livers of cystathionine β-synthase deficient mice having HH.5 This effect likely involves, in part, the thiol group of homocysteine because other thiol-containing agents show similar but less dramatic effects on mitochondrial mRNA levels. These findings suggest that homocysteine causes minor mitochondrial damage and/or turnover to which cells respond by increasing mitochondrial gene expression, thereby maintaining growth and viability. In support of this concept, the homocysteine-induced expression of NAD-dependent methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase in human vascular ECs (28) is associated with increased mitochondrial biogenesis (29).

Increased mitochondrial RNA levels may also reflect a cellular response involving either the metabolism of homocysteine or its removal from the cell, both of which require cellular ATP. It is well established that the mitochondrial folate metabolism pathway is an important component of the homocysteine remethylation pathway (1–4, 30). Thus, an increase in intracellular levels of homocysteine, either endogenously (formed in the methionine biosynthetic pathway) or exogenously (added to the culture medium), would be expected to activate genes encoding enzymes within this pathway. The finding that homocysteine induces the expression of NAD-dependent methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase, a bifunctional mi
mitochondrial enzyme involved in homocysteine remethylation (28), supports this concept. Because the mitochondrial folate and homocysteine remethylation pathways require cellular ATP, increased intracellular levels of homocysteine would be expected to deplete cellular ATP levels. This is consistent with recent findings indicating that overexpression of SAM in Chinese hamster ovary cells causes depletion of ATP (31). Depletion of cellular ATP could also result from the conversion of excess intracellular homocysteine to homocysteine thiolactone, an ATP-dependent reaction catalyzed by methionyl-tRNA synthase (32). Thus, as homocysteine accumulates within the cell, a futile cycle of synthesis and hydrolysis of homocysteine thiolactone occurs, which could potentially deplete intracellular ATP levels. To compensate for this decrease, cells would need to generate more ATP, presumably by increasing both mitochondrial activity and gene expression. This is consistent with our observation that homocysteine induces the expression of CO3/ATPase 6,8 and ND5, both of which are components of the respiratory transport chain.

Earlier in vitro studies have demonstrated that Cu²⁺ or ceruloplasmin catalyzes the auto-oxidation of homocysteine, thereby generating H₂O₂, increasing intracellular levels of homocysteine, and leading to cell injury (15–17). Because catalase prevents homocysteine-induced cell injury, it has been suggested that cell injury is largely due to H₂O₂ and not homocysteine. Our findings also confirm the importance of H₂O₂; however, we support a mechanism whereby homocysteine and H₂O₂ act synergistically to cause mitochondrial damage and to decrease cell growth and viability. Thus, we have shown that homocysteine in the presence of Cu²⁺ causes severe changes in mitochondrial ultrastructure, decreases mitochondrial RNA levels (marker of mitochondrial damage) and inhibits both cell growth and mitochondrial respiration rates. Furthermore, intracellular levels of H₂O₂ are significantly increased in the presence of homocysteine and Cu²⁺, and homocysteine together with H₂O₂, but not H₂O₂ alone, decreases mitochondrial RNA levels. The observation that catalase, but not free radical scavengers, blocks the decrease in mitochondrial RNA levels and prevents changes in mitochondrial ultrastructure indicates that this effect is specific for H₂O₂ and not superoxide anions or hydroxyl radicals. These findings are also consistent with previous studies demonstrating that extracellularly generated free radicals are unlikely to contribute to homocysteine-induced cytotoxicity (33). Whether these increased intracellular levels of H₂O₂ directly cause mitochondrial damage or lead to an increase in intracellular free radicals, which have been shown to play a role in homocysteine-induced cytotoxicity (33), is not known. However, the observation that H₂O₂ fails to increase mitochondrial damage and does not inactivate cytochrome c oxidase (34) suggests the involvement of intracellular...
Free radicals generated from H$_2$O$_2$. How homocysteine and H$_2$O$_2$ act together to enhance mitochondrial damage is not entirely clear; however, several possibilities exist. The fact that homocysteine does not elicit an oxidative stress response (18) and decreases the activity of several antioxidant enzymes both in vitro and in vivo (18, 19, 35) suggests that homocysteine could potentially enhance the susceptibility of cells to H$_2$O$_2$. This is particularly relevant because heat shock pretreatment, which induces the expression of HSPs and is known to protect mitochondria from H$_2$O$_2$-induced cell injury (25), prevents the decrease in mitochondrial RNA levels caused by homocysteine and Cu$^{2+}$. The ability of homocysteine to deplete cellular levels of NAD and ATP could potentially lead to de-energization of mitochondria, resulting in increased susceptibility to oxidative stress. Indeed, overexpression of SAM in Chinese hamster ovary cells leads to NAD and ATP depletion and enhanced H$_2$O$_2$-induced oxidative injury (31). Alternatively, homocysteine could potentially affect bcl-2 expression or cause cytochrome c release from mitochondria, key components involved in mitochondria-mediated cell death (36).

Our findings, as well as those of others (14–17, 33), suggest that homocysteine-induced cell toxicity is dependent on H$_2$O$_2$. Although the relationship between homocysteine-induced vascular disease and H$_2$O$_2$ remains unknown, recent studies suggest that H$_2$O$_2$ likely plays a role in the progression of vascular disease and that homocysteine may accelerate this process. Thus, the expression and synthesis of extracellular superoxide dismutase are increased in macrophages within atherosclerotic vessels, which could lead to an accumulation of extracellular H$_2$O$_2$ (37). Furthermore, rabbits fed a high-methionine diet, which leads to HH, have elevated levels of aortic superoxide dismutase, as well as disturbances in lipid peroxidation and antioxidant processes (35). Whether homocysteine directly alters the expression of superoxide dismutase in these macrophages and/or enhances the effects of H$_2$O$_2$ in atherosclerotic vessels remains to be determined.

In summary, these studies demonstrate that homocysteine alters mitochondrial gene expression, structure, and function. In addition, homocysteine and H$_2$O$_2$ act synergistically to pro-

**Fig. 8.** Ultrastructural analysis of DAMI cells demonstrating homocysteine-induced changes in mitochondria. Transmission electron microscopy of control DAMI cells (A), DAMI cells exposed to 1 mM homocysteine for 18 h (B), DAMI cells exposed to 1 mM homocysteine and 4 μM Cu$^{2+}$ for 18 h (C), or DAMI cells exposed to 1 mM homocysteine, 4 μM Cu$^{2+}$, and 2500 units/ml catalase for 18 h (D). m, mitochondria; v, intracytoplasmic vesicles; er, endoplasmic reticulum. Magnification, × 5000.

**Fig. 9.** Homocysteine and Cu$^{2+}$ increase intracellular levels of H$_2$O$_2$. Following a 60-min pulse with 100 μM DCF-diace tate, DAMI cells were treated for 1 h in the absence (control) or presence of either 4 μM Cu$^{2+}$, 1 or 5 mM homocysteine (Hcy), 1 or 5 mM homocysteine and 4 μM Cu$^{2+}$, or 1 mM H$_2$O$_2$. DCF fluorescence was determined for each sample by flow cytometry as described under “Experimental Procedures.” Results are represented as percentage of control and are the means ± S.E. of four separate cell experiments. *, p < 0.01 versus control cells.
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Fig. 10. Precursors of intracellular glutathione (A), but not free radical scavengers (B), block the decrease in CO3/ATPase 6,8 mRNA levels in DAMI cells treated with homocysteine and Cu2+. Total RNA (10 μg/lane) isolated from DAMI cells cultured for 18 h was analyzed by Northern hybridization using a radiolabeled CO3/ATPase 6,8 cDNA probe (upper panel). Control for RNA loading and integrity was assessed by ethidium bromide staining of the 28S and 18S rRNA bands (lower panel).

Fig. 11. Preexposure to heat shock blocks the decrease in CO3/ATPase 6,8 and HSP60 mRNA levels in DAMI cells treated with homocysteine and Cu2+. DAMI cells were preexposed for 2 h at 42 °C and allowed to recover for 6 (lanes 4 and 5) or 18 (lanes 6 and 7) h at 37 °C. Following recovery, cells were exposed to homocysteine and Cu2+ for 18 h, and total RNA was isolated and analyzed by Northern hybridization using radiolabeled cDNA probes to either CO3/ATPase 6,8 (A) or HSP60 (B) (upper panel). Control for RNA loading and integrity was assessed by ethidium bromide staining of the 28S and 18S rRNA bands (lower panel).

TABLE III
Intracellular GSH levels in DAMI cells treated for 4 h with either homocysteine or NAC

| Treatment     | GSH (nmol/10^6 cells) | % of control |
|---------------|-----------------------|-------------|
| Control       | 0.472 ± 0.011         |             |
| 1 mM homocysteine | 0.710 ± 0.023       | 50          |
| 10 mM homocysteine | 1.065 ± 0.027        | 113         |
| 1 mM NAC      | 0.676 ± 0.016         | 43          |
| 10 mM NAC     | 0.928 ± 0.008         | 97          |

*p < 0.01 versus control and 1 mM homocysteine.

Remote mitochondrial damage, possibly by increasing the susceptibility of cells to oxidative stress. The recent observation that overexpression of SAM in Chinese hamster ovary cells enhances H2O2-induced oxidative injury (31) supports this concept and suggests an intricate relationship among homocysteine metabolism, oxidative stress, and mitochondrial function. Although our findings provide evidence for homocysteine-induced mitochondrial damage in DAMI cells, it is unclear whether this occurs in all cell types. Based on the variability in homocysteine metabolism (32), mitochondrial activity (38, 39), and antioxidant status (40) among mammalian cells in vitro and in vivo, it is likely that differences in susceptibility exist. Further elucidation of the mechanism by which homocysteine and H2O2 causes mitochondrial damage may allow for a better understanding of the cytotoxic effects elicited by this physiologically relevant thiol-containing amino acid.

Acknowledgment—We thank Dr. Jack Hirsh for critical reading of the manuscript.

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