Endothelial Cell Injury Due to Copper-catalyzed Hydrogen Peroxide Generation from Homocysteine

Gordon Starkebaum and John M. Harlan
Department of Medicine, Veterans Administration Medical Center and Harborview Medical Center, Divisions of Rheumatology and Hematology, Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195

Abstract

We have examined whether the toxic effects of homocysteine on cultured endothelial cells could result from the formation and action of hydrogen peroxide. In initial experiments with a cell-free system, micromolar amounts of copper were found to catalyze an oxygen-dependent oxidation of homocysteine. The molar ratio of homocysteine oxidized to oxygen consumed was ~4.0, which suggests that oxygen was reduced to water. The addition of catalase, however, decreased oxygen consumption by nearly one-half, which suggests that H_2O_2 was formed during the reaction. Confirming this hypothesis, H_2O_2 formation was detected using the horseradish peroxidase-dependent oxidation of fluorescent scopoletin. Ceruloplasmin was also found to catalyze oxidation of homocysteine and generation of H_2O_2 in molar amounts equivalent to copper sulfate. Finally, homocysteine oxidation was catalyzed by normal human serum in a concentration-dependent manner.

Using cultured human and bovine endothelial cells, we found that homocysteine plus copper could lyse the cells in a dose-dependent manner, an effect that was completely prevented by catalase. Homocystine plus copper was not toxic to the cells. Specific injury to endothelial cells was seen only after 4 h of incubation with homocysteine plus copper. Confirming the biochemical studies, ceruloplasmin was also found to be equivalent to Cu^{++} in its ability to cause injury to endothelial cells in the presence of homocysteine.

Since elevated levels of homocysteine have been implicated in premature development of atherosclerosis, these findings may be relevant to the mechanism of some types of chronic vascular injury.

Introduction

Patients with inborn errors of methionine metabolism that result in homocystinuria suffer from recurrent arterial and venous thrombosis (1–3). Of particular interest is the premature development of arterial atherosclerosis in these patients (1, 4). That elevated levels of homocysteine can induce vascular injury has been shown in several model systems. For example, animals parenterally given large doses of methionine, which leads to increased plasma homocysteine levels, develop atherosclerosis and pulmonary embolism (5). Harker et al. (6, 7) found that chronic infusion of homocysteine into baboons induced patchy desquamation of endothelial cells in large arteries, release of endothelial cells into circulation, and increased platelet consumption. All homocysteinemic baboons developed typical atherosclerotic lesions. In vitro studies using cultured endothelial cells further support the hypothesis that homocysteine may be toxic to the endothelial cells (8, 9).

The mechanism whereby homocysteine can injure endothelial cells, however, is not well understood. Wall et al. (9) found that catalase could prevent homocysteine-induced endothelial cell injury in vitro, suggesting that hydrogen peroxide was the mediator. Since copper-catalyzed oxidation of other thiols can lead to reduction of oxygen with generation of hydrogen peroxide (10–12), we have examined the possibility that a similar process could occur with homocysteine. The results indicate that copper catalyzes an oxygen-dependent oxidation of homocysteine and that during the reaction hydrogen peroxide is generated. Lysis of cultured endothelial cells follows incubation with homocysteine plus copper but can be completely prevented by catalase.

Methods

Chemicals and buffers. Homocysteine, homocystine, scopoletin, glucose oxidase, superoxide dismutase, catalase, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB),[1] and human ceruloplasmin, type X were purchased from Sigma Chemical Co. (St. Louis, MO). Ceruloplasmin was further purified by sequential chromatography over Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ), to obtain the monomeric protein, followed by Chelex 100 (Bio-Rad Laboratories, Richmond, CA) to remove free copper. Cupric sulfate and ferrous sulfate were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ) and horseradish peroxidase was from Worthington Diagnostics Div., Millipore Corp. (Freehold, NJ). Reagents were freshly prepared in phosphate-buffered saline (PBS) and kept in a sealed tube on ice until used. The composition of balanced salt solution (BSS) used for the cell-free experiments was as follows: KCl, 0.4 g/liter; $K_2H_2PO_4$, 0.06 g/liter; $MgCl_2 \cdot 6H_2O$, 0.1 g/liter; NaCl, 8.0 g/liter; $NaHCO_3$, 0.35 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 0.09 g/liter; glucose, 1.0 g/liter; $CaCl_2 \cdot 6H_2O$, 0.147 g/liter, pH 7.4. Normal human serum was pooled from three healthy individuals and was stored at −20°C until used.

Oxidation of homocysteine. Loss of reduced sulfhydryl (SH) was measured with Ellman's reagent (DTNB) as previously described (13). For experiments performed in the presence of serum, the reaction of DTNB with serum SH groups was corrected for at each concentration. No oxidation of serum SH groups was observed during the 10-min incubation period.

Oxygen consumption. These studies were performed with a Clark oxygen electrode and oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) in a volume of 3.0 ml as previously described (14). The results are expressed as micromoles/liter of oxygen consumed per unit time. In experiments performed in the presence of serum a low level but dose-dependent consumption of oxygen was noted during the 10-min reaction with serum alone (maximum O_2 consumption with

Address correspondence to Dr. Starkebaum, Medical Service (111), Veterans Administration Medical Center, 1660 S. Columbian Way, Seattle, WA 98108.

Received for publication 28 May 1985 and in revised form 4 November 1985.

The Journal of Clinical Investigation, Inc.
Volume 77, April 1986, 1370–1376

1. *Abbreviations used in this paper:* BSS, balanced salt solution; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent); HPO, horseradish peroxidase.

100% serum, 12.4±2.9 μM/10 min, mean±1 SD, n = 3). Therefore, oxygen consumption due to serum alone was subtracted from each result obtained in the presence of homocysteine plus serum.

Hydrogen peroxide generation. The horseradish peroxidase (HPO)-dependent oxidation of scopoletin was measured using a fluorescent spectrophotometer (model MPF-2A; Perkin-Elmer Corp., Norwalk, CT) as previously described (15). Except as noted, all assays were performed in 1 × 1-cm cuvettes containing 2.5 ml BSS, 4 μM scopoletin, and 2.2 μM HPO at 37°C. The initial rate of H_2O_2 generation was determined immediately after mixing all reagents in the cuvette. For some experiments, to determine the amount of accumulated H_2O_2 during the reaction, scopoletin and HPO were added after 3 min and the immediate loss of fluorescence was recorded.

Cell culture. Human umbilical vein and bovine aortic endothelial cells were isolated by collagenase treatment of vessels as previously described (16). Human aortic smooth muscle cells were a gift from Dr. Russell Ross, University of Washington, Seattle, WA, and were isolated from vessel explants as previously described (17). Human endothelial cells were maintained in 20% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) and the bovine endothelial cells and human smooth muscle cells in 10% FCS in either RPMI 1640 (human umbilical vein cells) or Waymouth's (bovine and human aortic cells) medium MB 752/1 (Gibco, Grand Island, NY). The human umbilical vein endothelial cells were used in first passage and the bovine aortic endothelial cells were used in 8th–16th passage. Human aortic smooth muscle cells were tested in fifth passage.

^{51}Cr-release assay. The ^{51}Cr-release assay was performed as previously described (16). Briefly, cells from the same strain and passage were plated in Falcon microtest II plates (Falcon Labware, Division of Becton-Dickinson & Co., Oxnard, CA) at a density of 10^5 cells/cm^2 providing a visually confluent monolayer after overnight incubation. ^{51}Chromium (^{51}Cr) as sodium chromate (1 mCi/ml in saline, 200–500 Ci/g, New England Nuclear, Boston, MA) was added at the time of plating at a concentration of 10 μCi/ml. After an 18–24-h incubation and labeling, the monolayers were washed with three rapid, successive well-volume exchanges of PBS with 10% FCS dispensed by pipette and removed by vacuum aspiration (5 psi). After the final wash, test medium was added to a final volume of 100 μl per well. Incubations were conducted for up to 6 h at 37°C in 5% CO_2. 100 μl of cell-free supernatant medium were removed for determination of specific ^{51}Cr-release, calculated as follows: A − B/C − B × 100%, where A represents the mean test ^{51}Cr cpm released, B represents the mean spontaneous ^{51}Cr-cpm released, and C represents the mean maximum ^{51}Cr-cpm released. Maximum ^{51}Cr-release was determined by incubation in 0.1% Triton X-100 (New England Nuclear). Spontaneous ^{51}Cr-release was determined in control monolayers incubated in Waymouth's medium with 10% FCS and was 5–15% of maximum ^{51}Cr-release after a 6-h incubation. Statistical significance was determined by comparing mean test and mean control ^{51}Cr-cpm released by two-tailed, unpaired t statistic.

Results

Influence of copper on homocysteine oxidation and oxygen consumption. In BSS treated with Chelex-100 to remove Cu^{++} or containing 1 mM EDTA, <3% of 500 μM homocysteine was oxidized at 37°C during exposure to air for up to 60 min (n = 3). Adding increasing amounts of Cu^{++} to homocysteine in BSS, however, caused oxidation of the thiol and consumption of oxygen in a dose-dependent fashion (Fig. 1, Fig. 2). Over the range of copper concentrations 1–50 μM, the molar ratio of homocysteine oxidized to oxygen consumed during a 10-min reaction was 3.68±0.56 (mean±1 SD, n = 16). A similar ratio was obtained with homocysteine concentrations over the range of 1–20 × 10^{-4} M in the presence of 1/20th (molar) copper. Copper in the form of ceruloplasmin also caused oxidation of homocysteine in concentrations similar to those found with

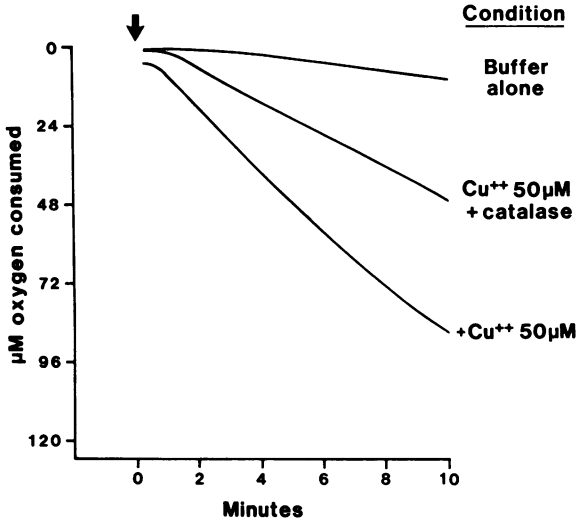

Figure 1. Consumption of oxygen seen with 500 μM homocysteine in BSS. Adding 50 μM Cu^{++} markedly increased the rate of O_2 consumption, whereas adding catalase (500 U/ml) to homocysteine plus Cu^{++} decreased the rate. Results from one experiment; similar results were obtained in five other experiments.

$CuSO_4$, assuming 6 mol of copper per mole of ceruloplasmin (Fig. 2).

To confirm that copper present in normal human serum could also catalyze oxidation of homocysteine, various concen-

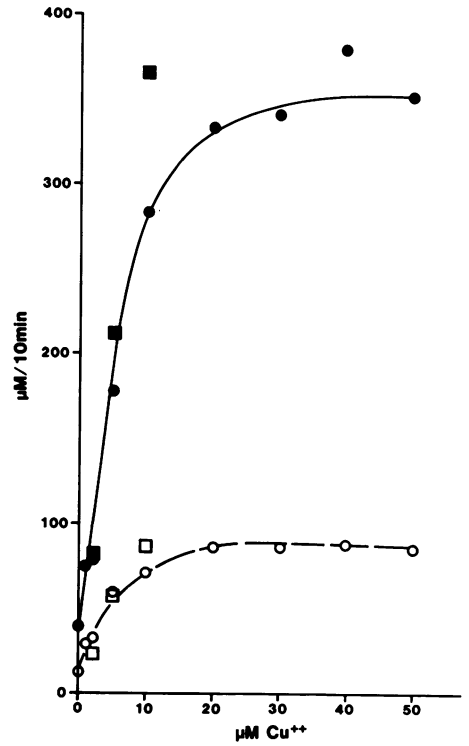

Figure 2. Influence of Cu^{++} on oxidation of 500 μM homocysteine (●) and oxygen consumption (○) during a 10-min reaction at 37°C in BSS. Ceruloplasmin also catalyzed oxidation of homocysteine (■) and O_2 consumption (□) in amounts equimolar to $CuSO_4$. Results from one experiment; similar results were obtained in three other experiments.

trations of serum in BSS were mixed with 500 μM homocysteine. The results indicate that when added to homocysteine, serum promoted consumption of oxygen and oxidation of the thiol in a dose-dependent fashion (Fig. 3). In this case, however, the molar ratio of homocysteine oxidized to oxygen consumed was higher than that seen in buffer alone (6.3±0.8, n = 5).

To confirm that oxygen was necessary for the copper-catalyzed oxidation of homocysteine, the reaction was carried out under nitrogen. In air, 60.7±9.7% of 500 μM homocysteine was oxidized in the presence of 50 μM Cu^{++} in 10 min (mean±1 SD, n = 3), whereas under N_2 <1% was oxidized ($P < 0.001$). These results indicate that Cu^{++} catalyzes an oxygen-dependent oxidation of homocysteine. Further experiments were then performed to determine whether oxygen was reduced to H_2O_2 during the reaction.

Generation of hydrogen peroxide. Initial evidence for generation of H_2O_2 was the finding that catalase (1,000 U/ml) decreased oxygen consumption in the presence of 500 μM homocysteine plus 10–50 μM Cu^{++} by 42.4±4.4% (n = 6, Fig. 1). Similarly, adding catalase decreased oxygen consumption in the presence of 500 μM homocysteine plus 75% normal human serum by 55.7±9.3% (n = 3), (Fig. 3). H_2O_2 generation was then measured by the HPO-mediated oxidation of fluorescent scopoletin as described previously (14, 15). For comparison, the generation of H_2O_2 by the glucose oxidase plus glucose system was also measured. In the absence of HPO, homocysteine failed to cause oxidation of scopoletin (not shown). In the presence of HPO, adding 50–500 μM homocysteine caused scopoletin to be oxidized in a dose-dependent fashion (Table I). Catalase inhibited the rate of scopoletin oxidation as did EDTA (Table I). Consistent with the latter finding, low levels of Cu^{++} (~0.5 μM) were found to be present in the reaction mixture by atomic absorption analysis. In contrast, EDTA augmented the rate of H_2O_2 generation seen with glucose oxidase; as expected, catalase decreased the rate (Table I).

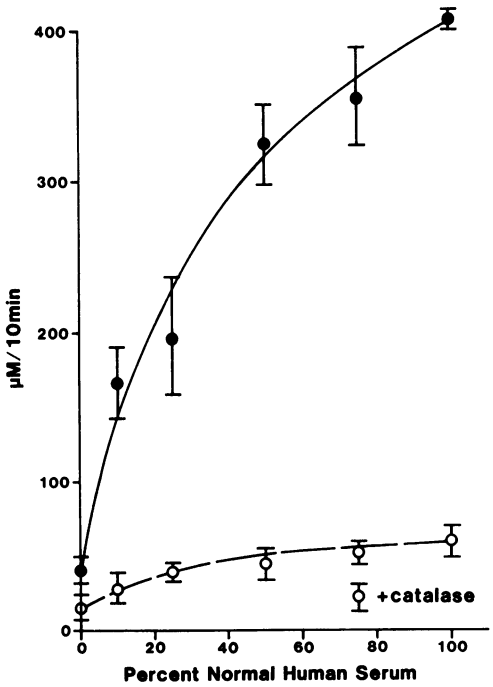

Figure 3. Influence of normal human serum on oxidation of 500 μM homocysteine (●) and oxygen consumption (○) during a 10-min reaction at 37°C in BSS. Catalase (500 U/ml) decreased O_2 consumption by ~50% (mean±1 SD, n = 3 for each point).

Table I. Generation of Hydrogen Peroxide by Homocysteine or by Glucose Oxidase plus Glucose

Condition	Initial rate of H_2O_2 generation, μM/min*	Micromoles/liter of H_2O_2 remaining after 3 min‡
	mean±1 SD (n)	*mean±1 SD (n)*
Glucose oxidase (0.32 μg/ml) + glucose (GO/G)	0.59±0.11 (10)	1.34±0.14 (12)
GO/G + catalase (500 U/ml)	0.33±0.05 (6)	0 (6)
GO/G + EDTA 1 mM	0.86±0.05 (3)	2.37±0.06 (3)
Homocysteine 50 μM	0.20±0.12 (6)	0 (3)
100 μM	0.56±0.07 (12)	0.20±0.18 (9)
500 μM	1.35±0.37 (6)	0 (3)
Homocysteine 100 μM + catalase	0.29±0.02 (6)	0.04±0.03 (6)
Homocysteine 100 μM + EDTA 1 mM	0.02±0.01 (6)	0 (3)
GO/G + Homocysteine 50 μM	0.49±0.10 (6)	0.53±0.28 (3)
100 μM	0.75±0.12 (6)	0.29±0.20 (3)
500 μM	1.42±0.48 (6)	0 (3)

* Measured immediately after adding to 4 μM scopoletin + 2.2 μM horseradish peroxidase in 2.5 ml balanced salt solution.
‡ Measured after 3 min of reaction by adding 4 μM scopoletin + 2.2 μM horseradish peroxidase and measuring immediate loss of fluorescence.

Finding a molar ratio of homocysteine oxidized to oxygen consumed of ~4.0 suggested that little H_2O_2 would accumulate during copper-catalyzed oxidation of homocysteine. To confirm this possibility, the amount of H_2O_2 that remained after a 3-min reaction was determined. The results indicate that virtually no H_2O_2 accumulated in the presence of 50–500 μM homocysteine (Table I). Furthermore, although H_2O_2 did accumulate in the presence of glucose oxidase, adding homocysteine to glucose oxidase decreased H_2O_2 remaining after 3 min in a dose-dependent fashion (Table I).

In other studies, adding 0.1–5.0 μM Cu^{++} to 250 μM homocysteine in the presence of 200 μM EDTA resulted in a dose-dependent increase in the rate of H_2O_2 generation (Fig. 4). Concentrations of Cu^{++} >5.0 μM, however, decreased formation of H_2O_2 as measured by this assay (Fig. 4). Copper in the form of ceruloplasmin also catalyzed generation of H_2O_2 in the presence of homocysteine in a manner nearly identical to that seen with $CuSO_4$ (Fig. 4).

Effect of homocysteine on cultured endothelial cells. In Waymouth's medium containing 10% FCS (Cu^{++}, <0.1 μM), homocysteine in concentrations up to 5 mM failed to induce lysis of cultured bovine or human endothelial cells in a 6-h incubation (data not shown). With the addition of 2 μM Cu^{++} to homocysteine, a time- and dose-dependent lysis of cultured bovine aortic endothelial cells was seen (Fig. 5), whereas homocysteine plus copper was without effect (Fig. 5). Copper in the form of ceruloplasmin was equally as effective as copper sulfate in promoting endothelial cell lysis by homocysteine (Fig. 6 *B*), but ferrous sulfate was not (Fig. 6 *A*). The lysis of endothelial cells

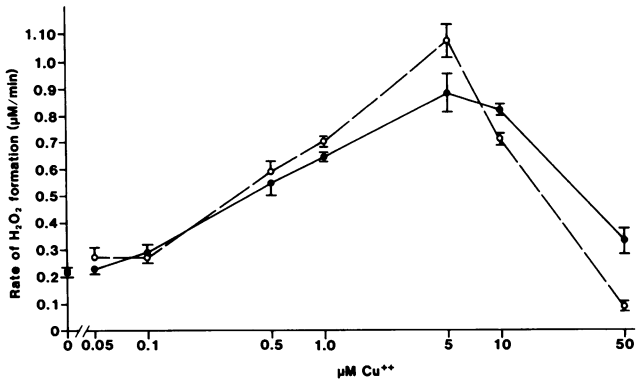

Figure 4. Effect of Cu^{++} (as copper sulfate) (●) or ceruloplasmin (○) on initial rate of H_2O_2 generation in BSS containing 200 μM EDTA with 250 μM homocysteine. The Cu^{++} concentration is shown on a log scale. Mean±1 SD from triplicate determinations in one experiment; similar results were obtained in two other experiments.

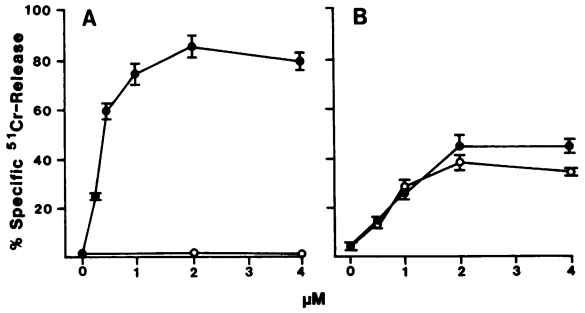

Figure 6. Role of copper (Cu^{++}) in homocysteine-mediated endothelial cell lysis. ^{51}Cr-labeled bovine aortic endothelial cells were incubated in 10% FCS in Waymouth's medium containing 500 μM homocysteine. In *A*, varying concentrations of Cu^{++} (as copper sulfate) (●) or Fe^{++} (as ferrous sulfate) (○) were added. In *B*, varying concentrations of Cu^{++} in the form of copper sulfate (●) or ceruloplasmin (○) were added. After 6 h incubation, aliquots of supernatant medium were removed for determination of specific ^{51}Cr-release. Values represent means±1 SE of six replicates.

by homocysteine and copper was prevented by the addition of catalase but not by boiled catalase or superoxide dismutase (Table II), which indicates that hydrogen peroxide or a hydrogen peroxide-derived product was a critical mediator of cell lysis. The cytolytic effect of homocysteine and copper and the protective effect of catalase were confirmed by examination of the monolayer by phase contrast microscopy (Fig. 7). Of note, both human and bovine endothelial cells were more susceptible to lysis by homocysteine and copper than were smooth muscle cells (Fig. 8).

Because the susceptibility of cultured endothelial cells to oxidant lysis differs between strain and passage number within the same strain (18), individual experiments were always performed with cells from the same strain and same passage. All experiments, however, were repeated with a different strain of endothelial cells. Other than minor differences in susceptibility to hydrogen peroxide-mediated lysis between strains (e.g., 80% lysis in Fig. 6 *A* vs. 40% lysis in Fig. 6 *B* after exposure to homocysteine, 500 μM, and Cu^{++}, 2 μM), similar results were obtained with the different strains.

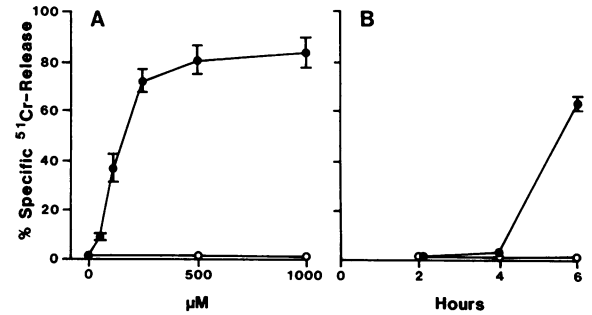

Figure 5. Time- and dose-dependent lysis of endothelial cells by homocysteine. ^{51}Cr-labeled bovine aortic endothelial cells were incubated in 10% FCS in Waymouth's medium containing 2 μM Cu^{++} (as copper sulfate) and varying concentrations of homocysteine (●) or homocystine (○). In *A* the incubation time was 6 h. In *B* the concentrations of homocysteine and homocystine were 500 μM. Aliquots of supernatant medium were removed for determination of specific ^{51}Cr-release. Values represent means±1 SE of six replicates.

Discussion

The results of this study confirm and extend those of Wall et al. (9) and suggest a mechanism whereby elevated levels of homocysteine could injure endothelial cells through copper-catalyzed generation of H_2O_2. The data indicate that in physiologic buffer copper catalyzes an oxygen-dependent reaction with homocysteine, leading to oxidation of the thiol and reduction of molecular oxygen. Our finding of ~4.0 mol of thiol oxidized for each mole of oxygen consumed in buffer suggests that the major reduction product of oxygen was water. This hypothesis was supported by the failure of H_2O_2 to accumulate during the brief reaction period. Furthermore, the results indicate that homocysteine itself can scavenge H_2O_2. Nevertheless, the finding that catalase decreased oxygen consumption seen with copper plus homocysteine by >40% suggested that H_2O_2 was formed during the reaction. Confirming this possibility, H_2O_2 was directly detected during copper-catalyzed oxidation of homocysteine by the HPO-catalyzed oxidation of scopoletin. The results

*Table II. Oxidative Lysis of Bovine Aortic Endothelial Cells**

Addition to endothelial monolayer	Percent specific ^{51}Cr-release
Catalase (1,000 U/ml)	0
Superoxide dismutase (200 U/ml)	0
Copper sulfate (4 μM)	0
Homocysteine (1 mM)	0
Homocysteine + copper sulfate	61±15
Homocysteine + copper sulfate + catalase	0
Homocysteine + copper sulfate + boiled catalase	84±7
Homocysteine + copper sulfate + superoxide dismutase	100%

* ^{51}Cr-labeled bovine aortic endothelial monolayers were incubated with control or test medium in 10% FCS in Waymouth's medium at 37°C. After a 6-h incubation at 37°C an aliquot of supernatant medium was removed for determination of specific ^{51}Cr-release. Values represent means±1 SE of eight replicates.

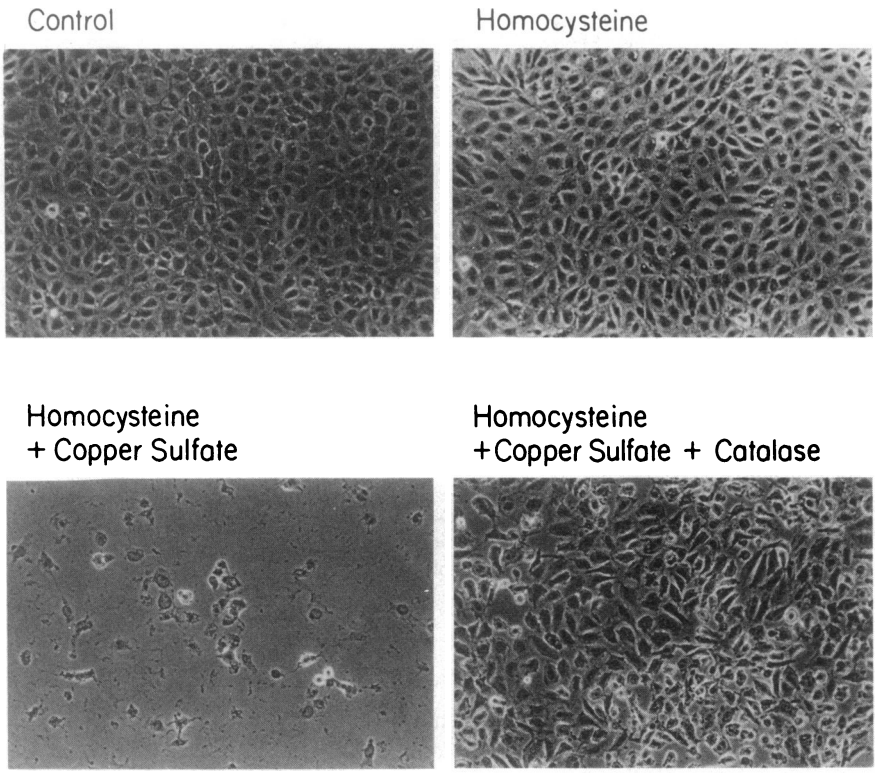

Figure 7. Phase contrast micrograph of bovine aortic endothelial cell monolayers incubated with homocysteine and copper sulfate. Bovine aortic endothelial cell monolayers were incubated for 6 h with 10% FCS in Waymouth's medium alone (control) or the same medium containing homocysteine (1000 μM) or homocysteine and copper sulfate (4 μM) or homocysteine and copper sulfate with catalase (2,500 U/ml). After incubation, the monolayers were washed once with Waymouth's medium and then fixed with 2% glutaraldehyde, 1% paraformaldehyde, 3% sucrose, and 0.001% $CaCl_2$ in 0.1 M cacodylate buffer. Magnification is approximately × 400.

indicate that low levels of copper increased the rate of H_2O_2 generation in a dose-dependent fashion, whereas EDTA markedly inhibited H_2O_2 generation. The decrease in H_2O_2 generation seen with higher Cu^{++} levels may have been the result of Cu^{++} catalyzed reduction of H_2O_2 to water.

Since the majority of plasma copper exists in the form of ceruloplasmin, its ability to catalyze oxidation of homocysteine was also examined. The results indicate that ceruloplasmin was capable of catalyzing oxidation of homocysteine and generation of H_2O_2 in concentrations that were approximately equimolar to those seen with Cu^{++}. Furthermore, normal human serum, which contains ~15 μM copper, primarily as ceruloplasmin (19), was also found to catalyze oxidation of homocysteine and oxygen consumption. The higher molar ratio of homocysteine oxidized to oxygen consumed seen in serum suggests the reaction may have been more complex than that in buffer alone. Although H_2O_2 formation was not directly measured in the presence of serum, the finding that catalase decreased oxygen consumption seen with homocysteine in the presence of 75% serum by nearly one half strongly suggests its formation.

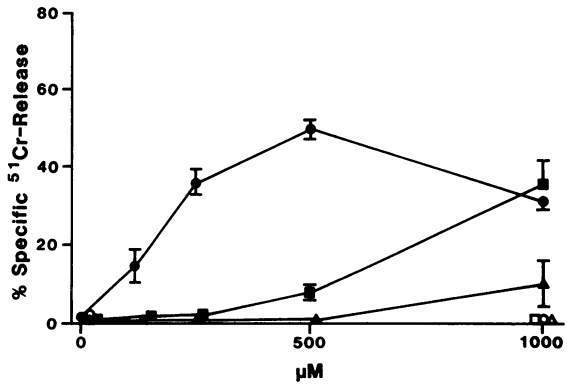

Figure 8. Comparison of homocysteine and copper (Cu^{++})-mediated lysis in bovine and human vessel wall cells. ^{51}Cr-labeled bovine aortic endothelial cells (●), human umbilical vein endothelial cells (■), and human aortic smooth muscle cells (▲) were incubated in 10% FCS in Waymouth's medium containing Cu^{++} 2 μM (as copper sulfate) and varying concentrations of homocysteine. Open symbols indicate incubations in the presence of catalase (1,000 U/ml). After 6 h incubation, aliquots of supernatant medium were removed for determination of specific ^{51}Cr-release. Values represent means±1 SE of six replicates.

It has been recognized for a number of years that copper catalyzes oxidation of various other thiols, in the process reducing molecular oxygen (10, 11, 19, 20). Several investigators have demonstrated superoxide (21), hydrogen peroxide (12), and hydroxyl radical generation (22) during thiol autoxidation. Furthermore, Chidambaram et al. (23) have recently shown that ceruloplasmin can also catalyze oxidation of cysteine with generation of superoxide and hydrogen peroxide. Reduced oxygen products generated during thiol oxidation have been shown to inhibit or lyse certain bacteria (24), hepatocytes (21), lymphocytes (14), and have been shown to cause mutations in bacteria (25).

Using cultured bovine aortic endothelial cells, homocysteine plus copper were found to lyse the cells in a dose-dependent fashion, an effect that was completely prevented by catalase but not by boiled catalase. Superoxide dismutase actually augmented the cytotoxic effects of copper plus homocysteine, possibly by virtue of the copper contained in the enzyme. In the presence of 2 μM copper, homocysteine concentrations as low as 100 μM were found to cause nearly 40% specific release of chromium 51. Also in the presence of 500 μM homocysteine, concentrations of copper >0.5 μM caused injury to endothelial cells. Confirming the biochemical studies, ceruloplasmin was also found to be equivalent to Cu^{++} in its ability to cause injury to endothelial cells in the presence of homocysteine. Specific ^{51}Cr-release was not seen before 4–6 h of incubation with homocysteine plus

copper. This time-course is similar to that found with another hydrogen peroxide-generating system, glucose plus glucose oxidase (18). Indeed, the kinetics of oxygen consumption and H_2O_2 generation observed with homocysteine plus copper closely resemble those seen with glucose plus glucose oxidase.

Two strains of human umbilical vein endothelial cells tested were more resistant to lysis by homocysteine and copper than several bovine aortic endothelial strains tested. Whether this reflects differences in species, vascular site, or passage number has not been determined. Also of note, human aortic smooth muscle cells were even more resistant to lysis by homocysteine and copper, an observation similar to that of Wall et al. (9). The biochemical basis for resistance to lysis by exogenously generated hydrogen peroxide is not known but may relate to differences in endogenous antioxidant enzymes (18) or to inherent resistance independent of such enzymes (26).

Substantial evidence implicates homocysteine as a factor in development of atherosclerosis (1–5). In addition to the finding that patients with homocystinuria develop premature atherosclerosis (1, 2), infusion of homocysteine into baboons results in endothelial damage and development of atherosclerotic plaques (6, 7). Deposition of platelets at sites of endothelial injury, leading to release of factors such as platelet-derived growth factor, which stimulate proliferation of vascular smooth muscle cells, may contribute to the pathogenesis of atherosclerosis (27). Untreated patients with homocystinuria have levels of homocysteine in the range of 100–200 μM, measured as the disulfides of homocyst(e)ine and homocysteine-cysteine (2, 3). Also, levels of ceruloplasmin are increased in these patients (28). These findings, together with our results, raise the possibility that vascular injury in patients with homocystinuria could be mediated by hydrogen peroxide generated by copper-catalyzed oxidation of homocysteine.

Abnormal methionine metabolism with appearance of increased levels of homocysteine or homocysteine-cysteine disulfide in circulation may reflect heterozygosity for cystathionine synthase deficiency and has been found in other groups of patients at risk for accelerated atherosclerosis. These include some patients with premature occlusive cerebrovascular and peripheral vascular disease (29, 30). Also, elevated plasma levels of homocysteine have been found in many normal males as well as postmenopausal females (31–33), and in patients with renal failure (34), including those on dialysis (35) and following renal transplantation (36). Levels of homocysteine found in plasma of these patients, however, are considerably lower than those found in homozygous homocysteinemic patients, e.g., 10–30 μM homocysteine. Whether such low levels of homocysteine can injure endothelial cells by generation of hydrogen peroxide is unknown. It is possible, however, that in these patients indirect effects of hydrogen peroxide may also play a role in the pathogenesis of vascular disease. These effects could include increasing serum levels of lipid hydroperoxide (37) with potentially important effects on arachidonate metabolism (38), inhibiting endothelial cell prostacyclin synthesis (39) or increasing oxidation of low density lipoproteins, contributing to increased uptake by arterial wall macrophages or smooth muscle cells (40). Further studies are needed to examine these possibilities.

Acknowledgments

The excellent technical assistance of Doug Webster, Penny Thompson, and Kathe Stanness is gratefully acknowledged. We thank Dr. Richard K. Root for his helpful suggestions and review of the manuscript, and Mrs. Maxine Pollock for typing it.

This work was supported by the Veterans Administration and by grant HL 18645 from the U. S. Public Health Service.

References

1. McCully, K. S. 1969. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am. J. Pathol.* 56: 111–128.

2. Grobe, H., and L. Balleisen. 1983. Thromboembolic vessel disease in homocystinuria. *In* The thromboembolic disorders. J. Van de Loo, C. R. M. Prentice, and F. K. Beller, editors. F. K. Schattauer Verlag, Stuttgart/New York. 513–522.

3. Mudd, S. H., and H. L. Levy. 1983. Disorders of transsulfuration. *In* The metabolic basis of inherited disease. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill, Inc., New York. 522–559.

4. Harker, L. A., S. J. Slichter, C. R. Scott, and R. Ross. 1974. Homocysteinemia. Vascular injury and arterial thrombosis. *N. Engl. J. Med.* 291:537–543.

5. McCully, K. S., and B. D. Ragsdale. 1970. Production of arteriosclerosis by homocysteinemia. *Am. J. Pathol.* 61:1–8.

6. Harker, L. A., R. Ross, S. J. Slichter, and C. R. Scott. 1976. Homocysteine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. *J. Clin. Invest.* 58:731–741.

7. Harker, L. A., J. M. Harlan, and R. Ross. 1983. Effect of sulfinpyrazone on homocysteine-induced endothelial injury and arteriosclerosis in baboons. *Circ. Res.* 53:731–739.

8. DeGroot, P. G., C. Willems, G. H. J. Boers, M. D. Gonsalves, W. G. Van Aken, and J. A. Van Mourik. 1983. Endothelial cell dysfunction in homocystinuria. *Eur. J. Clin. Invest.* 13:405–410.

9. Wall, R. T., J. M. Harlan, L. A. Harker, and G. E. Striker. 1980. Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thromb. Res.* 18:113–121.

10. Cavallini, D., C. Demarco, S. Dupre, and G. Rofilio. 1969. The copper catalyzed oxidation of cysteine to cystine. *Arch. Biochem. Biophys.* 130:354–361.

11. Jocelyn, P. C. 1972. Biochemistry of the SH group. The occurrence, chemical properties, metabolism and biological function of thiols and disulfides. Academic Press, Inc., New York. 94–115.

12. Zwart, J., H. M. C. Van Wolput, J. C. M. Van Der Cammen, and D. C. Konigsberger. 1981. Accumulation and reactions of H_2O_2 during the copper ion catalyzed auto oxidation of cysteine in alkaline medium. *J. Mol. Catal.* 11:69–82.

13. Sedlak, J., and R. H. Lindsay. 1968. Estimation of total protein-bound and nonprotein-bound sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25:192–205.

14. Starkebaum, G., and R. K. Root. 1985. D-penicillamine: analysis of the mechanism of copper-catalyzed hydrogen peroxide generation. *J. Immunol.* 134:3371–3378.

15. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* 53:945–955.

16. Harlan, J. M., P. D. Killen, L. A. Harker, and G. E. Striker. 1981. Neutrophil-mediated endothelial injury in vitro. Mechanisms of cell detachment. *J. Clin. Invest.* 68:1394–1403.

17. Ross, R., and J. A. Glomset. 1973. Atherosclerosis and the arterial smooth muscle cell. *Science (Wash. DC).* 180:1332–1339.

18. Harlan, J. M., J. D. Levine, K. S. Callahan, B. S. Schwartz, and L. A. Harker. 1984. Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. *J. Clin. Invest.* 73:706–713.

19. Ettinger, M. J. 1984. Copper metabolism and diseases of copper metabolism. *In* Copper proteins and copper enzymes, vol. III. R. Lontie, editor. CRC Press, Inc., Boca Raton, FL. 175–180.

20. Michaelis, L., and E. S. Guzman Barron. 1929. Oxidation-re-

duction systems of biological significance. II. Reducing effect of cysteine induced by free metals. *J. Biol. Chem.* 81:29–40.

21. Misra, H. P. 1974. Generation of superoxide free radical during the autoxidation of thiols. *J. Biol. Chem.* 249:2151–2155.

22. Saez, G., P. J. Thornalley, H. A. O. Hill, R. Hems, and J. V. Bannister. 1982. The production of free radicals during autoxidation of cysteine and their effect on isolated rat hepatocytes. *Biochim. Biophys. Acta.* 719:24–31.

23. Chidambaram, M. V., A. Zgirski, and E. Frieden. 1984. The reaction of cysteine with ceruloplasmin copper. *J. Inorg. Biochem.* 21: 227–239.

24. Nyberg, G. K., G. P. D. Granberg, and J. Carlsson. 1979. Bovine superoxide dismutase and copper ions potentiate the bactericidal effect of autoxidizing cysteine. *Appl. Environ. Microbiol.* 38:29–34.

25. Glatt, H., M. Protic-Sabljic, and F. Oesch. 1983. Mutagenicity of glutathione and cysteine in the Ames test. *Science (Wash. DC).* 220: 961–963.

26. O'Donnell-Tormey, J., C. J. DeBoer, and C. F. Nathan. 1985. Resistance of human tumor cells in vitro to oxidative cytolysis. *J. Clin. Invest.* 76:80–86.

27. Ross, R., and J. A. Glomset. 1976. The pathogenesis of atherosclerosis. *N. Engl. J. Med.* 295:369–377, 420–425.

28. Dudman, N. P. B., and D. E. L. Wilcken. 1983. Increased plasma copper in patients with homocystinuria due to cystathionine β-synthase deficiency. *Clinica. Chim. Acta.* 127:105–113.

29. Brattstrom, L. E., J. E. Hardebo, and B. L. Hultberg. 1984. Moderate homocysteinemia—a possible risk factor for arteriosclerotic cerebrovascular disease. *Stroke.* 15:1012–1016.

30. Boers, G. H. J., A. G. H. Smals, F. J. M. Trijbels, B. Fowler, J. A. J. M. Bakkeren, H. C. Schoonderwaldt, W. J. Kleijer, and P. W. C. Kloppenborg. 1985. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. *N. Engl. J. Med.* 313: 709–715.

31. Wilcken, D. E. L., and V. J. Gupta. 1979. Cysteine-homocysteine mixed disulphide: differing plasma concentrations in normal men and women. *Clin. Sci.* 57:211–215.

32. Gupta, V. J., and D. E. L. Wilcken. 1978. The detection of cysteine-homocysteine mixed disulphide in plasma of normal fasting man. *Eur. J. Clin. Invest.* 8:205–207.

33. Boers, G. H., A. G. Smals, F. J. Trijbels, A. I. Leermakers, and P. W. Kloppenborg. 1983. Unique efficiency of methionine metabolism in premenopausal women may protect against vascular disease in the reproductive years. *J. Clin. Invest.* 72:1971–1976.

34. Wilcken, D. E. L., and V. J. Gupta. 1979. Sulphur containing amino acids in chronic renal failure with particular reference to homocystine and cysteine-homocysteine mixed disulphide. *Eur. J. Clin. Invest.* 9:301–307.

35. Wilcken, D. E. L., V. J. Gupta, and S. G. Reddy. 1980. Accumulation of sulphur-containing amino acids including cysteine-homocysteine in patients on maintenance hemodialysis. *Clin. Sci.* 58:427–430.

36. Wilcken, D. E. L., V. J. Gupta, and A. K. Betts. 1981. Homocysteine in the plasma of renal transplant recipients: effects of cofactors for methionine metabolism. *Clin. Sci.* 61:743–749.

37. Warso, M. A., and W. E. M. Lands. 1985. Presence of lipid hydroperoxide in human plasma. *J. Clin. Invest.* 75:667–671.

38. Taylor, L., M. J. Menconi, and P. Polger. 1983. The participation of hydroperoxides and oxygen radicals in the control of prostaglandin synthesis. *J. Biol. Chem.* 258:6855–6857.

39. Whorton, A. R., M. E. Montgomery, and R. S. Kent. 1985. Effect of hydrogen peroxide on prostaglandin production and cellular integrity in cultured porcine aortic endothelial cells. *J. Clin. Invest.* 76:295–302.

40. Heinecke, J. W., H. Rosen, and A. Chait. 1984. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* 74:1890–1894.