Investigation of the inhibitory effects of homocysteine and copper on nitric oxide-mediated relaxation of rat isolated aorta

²Alison M. Emsley, ²Jamie Y. Jeremy, ²Guimar N. Gomes, ²Gianni D. Angelini & ^{*1}Frances Plane

¹Department of Pharmacology, University of Bristol, University Walk, Bristol, BS8 1TD, England, U.K.; ²Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol, BS2 8HW, England, U.K.

1 Elevated plasma levels of homocysteine (HC) and copper have both been associated with the development of inflammatory vascular diseases, such as atherosclerosis. In this study, the effects of a combination of HC and copper on nitric oxide (NO)-mediated relaxation of isolated rat aortic rings were investigated.

2 Exposure to HC (10–100 μ M; 30 min) had no effect on relaxation to acetylcholine (ACh; 0.01–10 μ M, $n=4$). Pre-incubation of aortic rings with a higher concentration of HC for an extended period (1 mM; 180 min) significantly inhibited endothelium-dependent relaxation ($n=4$), but this inhibition was prevented by the presence of the copper chelator bathocuprione (10 μ M, 180 min, $n=6$).

3 Exposure to HC (100 μ M) and copper (10–100 μ M; 30 min) caused a copper concentration-dependent inhibition of endothelium-dependent relaxation ($n=4$). This inhibitory effect was reduced in the presence of either superoxide dismutase (SOD; 100 u ml⁻¹; $n=4$) or catalase (100 u ml⁻¹; $n=4$), and further reduced by the presence of both enzymes ($n=5$).

4 HC and copper (100 μ M; 30 min) significantly inhibited endothelium-independent relaxation to glyceryl trinitrate (0.01–10 μ M; $n=8$). In contrast, HC (1 mM), alone or in combination with copper (100 μ M), did not inhibit relaxation to the endothelium-independent relaxant sodium nitroprusside (0.01–10 μ M; $n=4$).

5 These data indicate that the presence of copper greatly enhances the inhibitory actions of HC on NO-mediated relaxation of isolated aortic rings. The reduction of inhibition by catalase and SOD indicates a possible role for copper-catalyzed generation of superoxide and hydrogen peroxide leading to an increased inactivation or decreased production of endothelium-derived NO.

Keywords: Homocysteine; copper; nitric oxide

Abbreviations: ACh, Acetylcholine; GTN, glyceryl trinitrate; H₂O₂, hydrogen peroxide; OH, hydroxyl radical; HC, homocysteine; NO, nitric oxide; ONOO⁻, peroxynitrite; S-NO-HC, S-nitroso-homocysteine; SNP, sodium nitroprusside; O₂⁻, superoxide; SOD, superoxide dismutase

Introduction

Elevated plasma levels of the sulphur-containing amino acid homocysteine (HC) are suggested to be a powerful independent risk factor for premature atherosclerosis and other inflammatory vascular diseases (Berwanger *et al.*, 1995; McCully, 1996). Vascular endothelial cell dysfunction is considered to be a major factor in the development of atherosclerosis (Ross, 1993), and recent studies have shown that mild to moderate elevations in plasma levels of HC (20–35 μ M) are associated with impaired endothelium-dependent arterial dilatation in both humans (Tawakol *et al.*, 1997; Woo *et al.*, 1997) and monkeys (Lentz *et al.*, 1996) *in vivo*.

A number of studies have demonstrated that HC can cause endothelial cell dysfunction (Harker *et al.*, 1976; Stamler *et al.*, 1993). HC-induced inhibition of the synthesis of endothelium-derived nitric oxide (NO), increased loss of NO *via* the generation of superoxide anion (O₂⁻) and the formation of peroxynitrite (ONOO⁻), have been suggested to contribute to the attenuation of endothelium-dependent dilatation observed in the presence of homocysteinaemia (Upchurch *et al.*, 1996). However, the mechanisms by which HC alters vascular reactivity have not been clearly defined.

Raised plasma levels of the essential metalloid element copper have also been reported in patients with homocysteinaemia (Dudman & Wilcken, 1983) and may be a contributory factor

in atherogenesis (Aalbers & Houtman, 1985; Iskra *et al.*, 1993). Copper can catalyze the oxidation of thiols leading to the reduction of oxygen and the generation of hydrogen peroxide (H₂O₂; Cavallini *et al.*, 1969). Furthermore, HC-induced endothelial cell injury *in vitro* can be reduced by catalase, a scavenger of H₂O₂, indicating that H₂O₂ may be the mediator of HC-induced cellular damage (Wall *et al.*, 1980; Starkebaum & Harlan, 1986).

However, the elevation of plasma copper levels associated with inflammatory disease has been suggested to be an important component of the physiological response to overcome inflammation (reviewed by Sorenson, 1989). Copper can convert H₂O₂ to water and oxygen, and reduce the toxic hydroxyl radical (OH) to hydroxide (Farhataziz & Ross, 1977).

We have recently demonstrated that copper augments NO-mediated smooth muscle relaxation (Plane *et al.*, 1997). In the present study, we investigated the effect of the interaction between HC and copper on NO-mediated relaxation in the rat isolated aorta. A preliminary account of this study has been presented to the British Pharmacological Society (Emsley *et al.*, 1997).

Methods

Male Wistar-Kyoto rats (250–350 g) were stunned and decapitated. The descending aorta was rapidly removed,

*Author for correspondence.

cleared of adhering fat and connective tissue and cut into cylindrical segments approximately 2 mm in length.

Tension studies

Aortic segments were mounted in organ baths containing Krebs solution, maintained at 37°C and gassed with 95% O₂/5% CO₂. Isometric tension was measured with Grass FTO3 force displacement transducers and data recorded on disc (MacLab). Tissues were set to a pre-determined optimal tension of 1.5 g and left to equilibrate for 30 min, after which time the tension was reset.

Aortic rings were pre-contracted with phenylephrine (1–3 µM). The concentration of phenylephrine was adjusted to give a similar level of tone in all experiments (approximately 1 g). The tissues were then relaxed with a bolus dose of acetylcholine (ACh; 5 µM) to assess the integrity of the endothelium. Following washout and equilibration, the rings were pre-contracted and relaxed with cumulative concentrations of the endothelium-dependent vasodilator, ACh (0.01–10 µM) or the endothelium-independent vasodilators, sodium nitroprusside (SNP; 0.01–10 µM) and glyceryl trinitrate (GTN; 0.01–10 µM) in the presence or absence of HC and/or copper (30–180 min). The responses to SNP and GTN were slower to develop than those to ACh taking approximately 18 and 16 min respectively to attain full relaxation as compared to around 13 min for ACh. In experiments carried out to investigate the role of O₂⁻ or H₂O₂ in the observed inhibitory effect of HC and copper, superoxide dismutase (SOD) and/or catalase were added together with the HC and copper.

Solutions and drugs

For tension studies, aortic rings were maintained in Krebs buffer (pH 7.4) of the following composition (mM): NaCl 119, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 2.5 and glucose 11, and containing 2.8 µM indomethacin. Acetylcholine chloride, bathocuproinedisulphonic acid (disodium salt), catalase (bovine liver), copper (II) chloride, D-L homocysteine, L-phenylephrine hydrochloride, sodium nitroprusside, superoxide dismutase (bovine erythrocytes) were all obtained from Sigma (Poole, Dorset, U.K.). Glyceryl trinitrate was obtained from Schwarz Pharma Ltd (Chesham, Bucks, U.K.).

Analysis of data

Relaxation of isolated aortic rings is expressed as per cent reversal of phenylephrine-induced tone. All results are expressed as means ± s.e.mean for *n* separate experiments. Comparisons were made using Student's *t*-test or ANOVA followed by Dunnett's *post hoc* test for multiple comparisons with the control, with rejection of the null hypothesis at the 5% level.

Results

Effect of copper on endothelium-dependent relaxation of isolated rat aortic rings

ACh (0.01–10 µM) evoked endothelium-dependent relaxation of isolated rat aortic rings pre-contracted with phenylephrine (1–3 µM). Pre-incubation of aortic rings with copper (100 µM; 30 min) significantly potentiated relaxation to the endothelium-dependent vasodilator, ACh. Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of copper (100 µM; 30 min) are shown in Figure 1.

Effect of HC on endothelium-dependent relaxation of isolated rat aortic rings

Pre-incubation of arterial rings with HC (10–100 µM) for 30 min had no significant effect on relaxation to ACh. Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of HC (100 µM; 30 min) are shown in Figure 2a.

Exposure of aortic rings to a higher concentration of HC (1 mM) for 30 or 180 min significantly attenuated relaxation to ACh, reducing the maximum response from 96 ± 4 and 92 ± 6%, to 68 ± 9 and 46 ± 7% (*n* = 4 in each case; *P* < 0.01), respectively.

Pre-incubation with superoxide dismutase (SOD; 100 u ml⁻¹) or catalase (100 u ml⁻¹) alone, or in combination for 3 h had no significant effect on relaxation to ACh (*n* = 4 in each case). However, the inhibitory effect of HC (1 mM; 180 min) was significantly reduced by the presence of either superoxide dismutase (SOD; 100 u ml⁻¹; *n* = 4) or catalase (100 u ml⁻¹; *n* = 4) alone, or in combination (*n* = 4). Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of HC, SOD and catalase are shown in Figure 2b and c.

The presence of the copper chelator, bathocuprione (10 µM) in the Krebs buffer had no significant effect on relaxation to ACh (*n* = 6). Pre-incubation for 180 min with HC (1 mM) in the presence of bathocuprione (10 µM) had no significant effect on relaxation to ACh (*n* = 6). Concentration-relaxation curves for ACh-evoked relaxation in the presence and absence of HC (1 mM) and bathocuprione (10 µM) are shown in Figure 2d.

Effect of a combination of HC and copper on endothelium-dependent relaxation of rat isolated aortic rings

In the presence of copper (10–100 µM), pre-incubation of aortic rings with HC (100 µM; 30 min), which alone had

Figure 1 Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the presence and absence of copper (100 µM; 30 min). All mean values are from four experiments with s.e.means shown by vertical bars. ***P* < 0.01, **P* < 0.05 compared with control.

no effect on endothelium-dependent relaxation, caused a copper concentration-dependent inhibition of relaxation to ACh. In the presence of HC and 10 μ M copper relaxation to ACh was unaltered ($n=4$). However, in the presence of HC and 20 μ M copper, relaxation to ACh was significantly inhibited and the maximum response reduced from 95 ± 2 to $51.3 \pm 6.0\%$ ($n=4$, $P<0.01$). In the presence of HC and 100 μ M copper, the maximal response to ACh

was reduced from 91 ± 5 to $27.9 \pm 13.1\%$ ($n=4$, $P<0.01$). Representative traces showing ACh-evoked relaxation of an aortic ring in the absence and presence of HC, alone and in combination with copper (100 μ M) are shown in Figure 3a. Mean concentration-response curves for ACh-evoked relaxation in the presence of HC (100 μ M) and copper (10, 20 and 100 μ M; 30 min) are shown in Figure 3b.

Figure 2 (a) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (100 μ M; 30 min). * $P<0.05$ compared with control. (b) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (1 mM; 180 min), together with catalase (100 u ml^{-1}) or SOD (100 u ml^{-1}). (c) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (1 mM; 180 min), together with catalase and SOD (100 u ml^{-1}). ** $P<0.01$ compared with control, * $P<0.05$ compared with control, # $P<0.05$ compared to relaxation in the presence of HC (100 μ M; 30 min), ## $P<0.01$ compared to relaxation in the presence of HC (100 μ M; 30 min). All values are means from four experiments with s.e.means shown by vertical bars. (d) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings in the presence of HC (1 mM) and bathocuprione (10 μ M; 180 min). All values are means from six experiments with s.e.means shown by vertical bars.

Figure 3 (a) Representative traces showing changes in tension elicited by ACh (0.01–10 μM) in a rat isolated aortic ring pre-contracted with phenylephrine (3 μM). (i) control (ii) in the presence of HC (100 μM) alone (iii) in the presence of HC together with copper (both 100 μM). (b) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (100 μM) and increasing concentrations of copper (10–100 μM). All values are means from four experiments with s.e.means shown by vertical bars. ** $P < 0.01$ compared with control.

Effect of HC and copper on endothelium-independent relaxation

Exposure to HC (10 μM –1 mM; 30 min) alone did not alter relaxation to the endothelium-independent relaxant SNP ($n=4$). Exposure of aortic rings to copper (100 μM ; 30 min) significantly potentiated relaxation to SNP and the combination of HC (1 mM) and copper (100 μM) caused a similar potentiation of relaxation to SNP as seen in the presence of copper alone. Concentration-relaxation curves for SNP-evoked relaxation in the presence and absence of HC and copper are shown in Figure 4a.

GTN evoked concentration-dependent relaxation of endothelium-denuded aortic rings. Pre-incubation with HC (100 μM) or copper (100 μM) for 30 min had no significant effect on relaxation to GTN ($n=4$). However, exposure of aortic rings to HC and copper together (both 100 μM ; 30 min) significantly inhibited relaxation to GTN reducing the maximum relaxation from 96 ± 2 to $32 \pm 5\%$ ($n=8$; $P < 0.01$). Concentration-relaxation curves for GTN-evoked relaxation in the presence and absence of HC and copper are shown in Figure 4b.

Effect of catalase and SOD on the inhibition of endothelium-dependent relaxation by HC and copper

The inhibition of ACh-stimulated relaxation observed in the presence of HC and copper (both 100 μM) together was significantly reduced by the presence of either catalase (100 u ml^{-1} ; $n=4$) or SOD (100 u ml^{-1} ; $n=4$). In the presence of both catalase and SOD, the inhibitory effect of the combination HC and copper was further reduced and relaxations to ACh were not significantly different from controls ($n=4$). Concentration-response curves for ACh-evoked relaxation in the presence and absence of HC and copper, SOD and catalase are shown in Figure 5a and b.

Discussion

The results of the present study demonstrate that copper causes a concentration-dependent enhancement of the inhibition of NO-mediated relaxation of isolated vascular tissue by HC. In the presence of copper, a short exposure to HC, which alone had no effect, significantly inhibited endothelium-dependent relaxation to ACh and endothelium-independent relaxation to GTN, without inhibiting relaxation to the endothelium-independent relaxant SNP.

Normal plasma levels of HC are in the range of 1–10 μM (Berwanger *et al.*, 1995) but in homocysteinaemic patients plasma HC levels of 100–500 μM have been detected (Malinow, 1994). In the present study, addition of HC alone, at a concentration of 100 μM , had no significant effect on endothelium-dependent relaxation. Similarly, a previous study demonstrated that brief exposure of endothelial cells to low concentrations of HC did not alter NO-mediated inhibition of platelet aggregation but in fact resulted in the formation of S-nitroso-homocysteine (S-NO-HC), a vasodilator and an inhibitor of platelet aggregation, which does not generate H_2O_2 (Stamler *et al.*, 1993). Thus, the formation of S-NO-HC may attenuate the potentially adverse effects of this amino acid and could contribute to the lack of inhibition of endothelium-dependent relaxation observed following a short exposure to HC in our experiments.

In contrast, exposure of isolated tissues to a higher concentration of HC (1 mM for 30–180 min) did cause an

Figure 4 (a) Mean concentration-response curves for SNP-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC (1 mM) and copper (100 μ M). All values are means from four experiments with s.e.means shown by vertical bars. $**P < 0.01$ and $*P < 0.05$ compared with control. (b) Mean concentration-response curves for GTN-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC and copper (both 100 μ M; 30 min). All values are means from eight experiments with s.e.means shown by vertical bars. $**P < 0.01$ and $*P < 0.05$ compared with control.

attenuation of endothelium-dependent relaxation. Similar results have recently been reported in other isolated vascular preparations (Lang *et al.*, 1997; Quéré *et al.*, 1997), and prolonged exposure of cultured endothelial cells to high concentrations of HC have been shown to progressively impair their ability to inhibit platelet aggregation (Stamler *et al.*, 1993). However, the inhibition of relaxation by 1 mM HC was prevented by the presence of the copper chelator, bathocuprione indicating that the effects of HC observed in this study and in previous investigations (Lang *et al.*, 1997) may in fact be due to the interaction of HC with trace amounts of

Figure 5 (a) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC and copper (both 100 μ M; 30 min), together with catalase (100 u ml^{-1}) or SOD (100 u ml^{-1}). (b) Mean concentration-response curves for ACh-evoked relaxation of rat isolated aortic rings, in the absence and presence of HC and copper (both 100 μ M; 30 min), together with catalase and SOD (both 100 u ml^{-1}). All values are means from 4–5 experiments with s.e.means shown by vertical bars. $**P < 0.01$ compared with control. $\#P < 0.05$ compared to relaxation in the presence of HC and copper (both 100 μ M; 30 min), $###P < 0.01$ compared to relaxation in the presence of HC and copper (both 100 μ M; 30 min).

copper present in the Krebs buffer used during the experiments. This observation raises the possibility that during prolonged exposure of arteries to HC, as occurs *in vivo*, only very low concentrations of copper may be required to interact with HC and cause vascular dysfunction.

In the present study, the inhibitory effects of high concentrations of HC on endothelium-dependent relaxation were reduced by SOD and/or catalase. Oxidation of thiols can lead to the generation of both O_2^- and H_2O_2 , (Loscalzo, 1996)

and HC inhibits glutathione peroxidase, which normally removes H_2O_2 (Upchurch *et al.*, 1997). Thus, the attenuation of inhibition by SOD and catalase implicates both O_2^- and H_2O_2 in HC-induced endothelial dysfunction as previously suggested (Starkebaum & Harlan, 1986; Upchurch *et al.*, 1996). These reactive species can directly damage endothelial cells to decrease the production of NO and the reaction of NO with O_2^- leading to the formation of ONOO^- may contribute to a decreased NO-mediated relaxation (Beckman & Koppenol, 1996).

Exposure to copper alone potentiates NO-mediated endothelium-dependent relaxation in the rat isolated aorta, indicating that copper may play a role in the modulation of NO production (Plane *et al.*, 1997). In the present investigation copper caused a concentration-dependent enhancement of the inhibition of NO-mediated relaxation caused by HC. Thus, under pathophysiological conditions, the interaction between HC and essential metalloelements such as copper, may contribute to the vascular dysfunction in homocysteinaemic patients (Lentz *et al.*, 1996; Tawakol *et al.*, 1997; Woo *et al.*, 1997).

The inhibitory effect of the combination of HC and copper on endothelium-dependent relaxation was partially reduced by catalase or SOD, and further reduced by the two enzymes together, indicating a role for the generation of H_2O_2 and O_2^- in this process. Similarly, a previous study has shown that in the presence of low levels of copper ($1-10 \mu\text{M}$), HC causes lysis of cultured endothelial cells, an effect which is blocked by catalase (Starkebaum & Harlan, 1986). *In vivo*, almost all plasma copper is bound to caeruloplasmin and the levels of ionically bonded or salt forms of copper in plasma are too small to be measured accurately (May *et al.*, 1976). However, a number of studies have indicated that protein-bound copper can catalyse reactions in a similar manner to free copper (Starkebaum & Harlan, 1986; Ehrenwald *et al.*, 1993) and the reaction between O_2^- and NO leads to the formation of ONOO^- which can release of copper ions from caeruloplasmin (Swain *et al.*, 1994).

These results indicate a role for both O_2^- and H_2O_2 in the inhibition by HC and copper of NO-induced relaxation. The

mechanisms of action of these reactive oxygen species are quite distinct. O_2^- destroys NO in the extracellular space, and thus reduces the amount of available NO to cause relaxation (Gryglewski *et al.*, 1986; Rubanyi & Vanhouette, 1986). This action can be reversibly inhibited by SOD. In contrast H_2O_2 is known to stimulate NO production (Schimizu *et al.*, 1994) but subsequently causes irreversible endothelial damage leading to reduced NO production. The action of H_2O_2 can be prevented by catalase but cannot be reversed once endothelial damage has occurred. As SOD and catalase cannot enter cells, but reduce the inhibition of ACh mediated relaxation by HC and copper, the reactive oxygen species generated from the interaction between HC and copper appear to inhibit NO-mediated relaxation in the extracellular space.

The combination of HC and copper significantly inhibited NO-mediated relaxation to the endothelium-independent relaxant, GTN. However, in the presence of copper, alone or with HC, relaxation to the endothelium-independent relaxant SNP was not inhibited but was slightly, but significantly potentiated. This observation may reflect the ability of copper to enhance the release of NO from SNP, analogous to the release of NO from nitrosothiols (McAninly *et al.*, 1993) which may mask the inhibitory effect of the combination of HC and copper.

In conclusion, this study indicates that although ionically bonded copper does not inhibit NO-mediated responses, the interaction between copper and HC inhibits endothelium-dependent arterial dilatation which may in turn contribute to the pro-atherogenic effects of this amino acid. At present, the only treatment for homocysteinaemia is the administration of folic acid, alone or with vitamin B_6 or B_{12} . The clinical benefit of this therapy is unproven and thus the development of future strategies could focus on the possible use of copper chelators and antioxidants for the treatment of this condition.

A.M. Emsley is supported by a British Heart Foundation studentship and F. Plane is a Wellcome Trust Career Development Fellow. We would like to thank Prof John R.J. Sorenson of the University of Arkansas, U.S.A., for helpful criticism and discussion during the preparation of this manuscript.

References

- AALBERS, T.H.G. & HOUTMAN, J.P.W. (1985). Relationship between trace elements and atherosclerosis. *Sci. Tot. Environ.*, **43**, 255–283.
- BECKMAN, J.S. & KOPPENOL, W.H. (1996). Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am. J. Physiol.*, **271**, C1424–C1437.
- BERWANGER, C.L., JEREMY, J.Y. & STANSBY, G.D. (1995). Homocysteine and vascular disease. *Br. J. Surg.*, **82**, 726–731.
- CAVALLINI, D., DEMARCO, C., DUPRE, S. & ROFILIO, G. (1969). The copper catalyzed oxidation of cysteine to cystine. *Arch. Biochem. Biophys.*, **130**, 354–361.
- DUDMAN, N.P.B. & WILCKEN, D.E.L. (1983). Increased plasma copper in patients with homocystinuria due to cystathionine β -synthase deficiency. *Clin. Chim. Acta.*, **127**, 105–113.
- EHRENWALD, E., CHISOLM, G.M. & FOX, P.L. (1994). Intact human caeruloplasmin oxidatively modifies low density lipoprotein. *J. Clin. Invest.*, **93**, 1493–1501.
- EMSLEY, A., PLANE, F., ANGELINI, G.D. & JEREMY, J.Y. (1997). Copper interacts with homocysteine to inhibit nitric oxide formation in the rat isolated aorta. *Br. J. Pharmacol.*, **122**, 47P.
- FARHATAZIZ & ROSS, A.B. (1977). Selected and specific rates of reactions of transients from water in aqueous solution III. Hydroxyl radical and perhydroxyl radical and their radical ions. Reference Data System, National Bureau of Standards, U.S. Department of Commerce, Washington D.C.
- GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelial-derived relaxing factor. *Nature*, **320**, 454–456.
- HARKER, L.A., ROSS, R., SLICHTER, S.J. & SCOTT, C.R. (1976). Homocysteine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. *J. Clin. Invest.*, **58**, 731–741.
- ISKRA, M., PATELSKI, J. & MAJEWSKI, W. (1993). Concentrations of calcium, magnesium, zinc and copper in relation to free fatty acids and cholesterol in serum of atherosclerotic men. *J. Trace Elem. Electrolytes Health Dis.*, **7**, 185–188.
- LANG, D., HUSSAIN, S.A. & LEWIS, M.J. (1997). Homocysteine inhibits endothelium-dependent relaxation in isolated rabbit aortic rings. *Br. J. Pharmacol.*, **120**, 145P.
- LENTZ, S.R., SOBEY, C.G., PIEGORS, D.J., BHOPATKAR, M.Y., FARACI, F.M., MALINOW, M.R. & HEISTAD, D.D. (1996). Vascular dysfunction in monkeys with diet-induced hyperhomocysteinemia. *J. Clin. Invest.*, **98**, 24–29.
- LOSCALZO, J. (1996). The oxidant stress of hyperhomocysteinemia. *J. Clin. Invest.*, **98**, 5–7.
- MALINOW, M.R. (1994). Homocyst(e)ine and arterial occlusive diseases. *J. Intern. Med.*, **236**, 603–617.

- MAY, P.M., LINDER, P.W. & WILLIAMS, D.R. (1976). Ambivalent effect of protein binding on computed distributions of metal ions complexed with ligands in blood plasma. *Experientia*, **32**, 1492–1493.
- MCANINLY, J., WILLIAMS, D.L.H., ASKEW, S.C., BUTLER, A.R. & RUSSELL, C. (1993). Metal-ion catalysis in nitrosothiol (RSNO) decomposition. *J. Chem. Soc. Commun.*, **23**, 1758–1759.
- MCCULLY, K.S. (1996). Homocysteine and vascular disease. *Nature Med.*, **2**, 386–389.
- PLANE, F., WIGMORE, S., ANGELINI, G.D. & JEREMY, J.Y. (1997). The role of copper in mediating nitric oxide and prostacyclin synthesis in the rat aorta. *Br. J. Pharmacol.*, **121**, 345–350.
- QUÉRÉ, I., HILLAIRES-BUYS, D., BRUNSCHWIG, C., CHAPAL, J., JANBON, C., BLAYAC, J.P., PETIT, P. & LOUBATIÈRES-MARIANI, M.M. (1997). Effects of homocysteine on acetylcholine- and adenosine-induced vasodilatation of pancreatic vascular beds in rats. *Br. J. Pharmacol.*, **122**, 351–357.
- ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature*, **362**, 801–809.
- RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Superoxide anions and hyperoxia inactivate endothelial-derived relaxing factor. *Am. J. Physiol.*, **250**, H822–H827.
- SHIMIZU, S., SAITOH, Y., YAMAMOTO, T. & MOMOSE, K. (1994). Stimulation by hydrogen-peroxide of L-arginine metabolism to L-citrulline coupled with nitric oxide synthesis in cultured endothelial cells. *Res. Commun. Chem. Path. Pharmacol.*, **84**, 315–329.
- SORENSEN, J.R.J. (1989). Copper complexes offer a physiological approach to treatment of chronic diseases. *Prog. Med. Chem.*, **26**, 437–568.
- STAMLER, J.S., OSBORNE, J.A., JARAKI, O., RABBANI, L.E., MULLINS, M., SINGEL, D. & LOSCALZO, J. (1993). Adverse vascular effects of homocysteine are modulated by endothelium derived relaxing factor and related oxides of nitrogen. *J. Clin. Invest.*, **91**, 308–318.
- STARKEBAUM, G. & HARLAN, J.M. (1986). Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J. Clin. Invest.*, **77**, 1370–1376.
- SWAIN, J.A., DARLEY-USMAR, V. & GUTTERIDGE, J.M.C. (1994). Peroxynitrite releases copper from caeruloplasmin: implications for atherosclerosis. *FEBS Letts.*, **342**, 49–52.
- TAWAKOL, A., OMLAND, T., GERHARD, M., WU, J.T. & CREAGER, M.A. (1997). Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation*, **95**, 1119–1121.
- UPCHURCH, G.R., WELCH, G.N., FABIAN, A.J., FREEDMAN, J.E., JOHNSON, J.L., KEANEY, J.F. & LOSCALZO, J. (1997). Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J. Biol. Chem.*, **272**, 17012–17017.
- UPCHURCH, G.R., WELCH, G.N. & LOSCALZO, J. (1996). Homocysteine, EDRF and endothelial function. *J. Nutr.*, **126**, 1290S–1294S.
- WALL, R.T., HARLAN, J.M., HARKER, L.A. & STRIKER, G.E. (1980). Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thromb. Res.*, **18**, 113–121.
- WOO, K.S., CHOOK, P., LOLIN, Y.I., CHEUNG, A.S.P., CHAN, L.T., SUN, Y.Y., SANDERSON, J.E., METREWELL, C. & CELERMAJER, D.S. (1997). Hyperhomocysteinemia is a risk factor for arterial endothelial dysfunction in humans. *Circulation*, **96**, 2542–2544.

(Received July 24, 1998

Revised November 9, 1998

Accepted November 19, 1998)