Free Radical Research, Vol. 35, pp. 499–505
Reprints available directly from the publisher
Photocopying permitted by license only

Is Homocysteine a Pro-oxidant?

BRUNO ZAPPACOSTA[a,*], ALVARO MORDENTE[b], SILVIA PERSICHILLI[a], ANGELO MINUCCI[a], PAOLA CARLINO[a], GIUSEPPE ETTORE MARTORANA[b], BRUNO GIARDINA[a] and PASQUALE DE SOLE[a]

[a]*Instituto di Chimica e Chimica Clinica and CNR Centro Chimica dei Recettori Università Cattolica, Rome, Italy;* [b]*Istituto di Chimica Biologica, Università Cattolica del S. Cuore, Rome, Italy*

Accepted by Professor J. V. B. Bannister

(Received 19 December 2000; In revised form 16 March 2001)

High plasma homocysteine concentrations have been found to be associated with atherosclerosis and thrombosis of arteries and deep veins. The oxidative damage mediated by hydrogen peroxide production during the metal-catalyzed oxidation of homocysteine is to date considered to be one of the major pathophysiological mechanisms for this association.

In this work, a very sensitive and accurate method was employed to measure the effective production of H_2O_2 during homocysteine oxidation. Furthermore, the interaction of homocysteine with powerful oxidizing species (hypochlorite, peroxynitrite, ferrylmyoglobin) was evaluated in order to ascertain the putative pro-oxidant role of homocysteine.

Our findings indicate that homocysteine does not produce H_2O_2 in a significant amount (1/4000 mole/mole ratio of H_2O_2 to homocysteine). Moreover, homocysteine strongly inhibits the oxidation of luminol and dihydrorhodamine by hypochlorite or peroxynitrite and rapidly reduces back ferrylmyoglobin, the oxidizing species, to metmyoglobin.

All these results should, in our opinion, lead to a rethinking of the commonly held view that homocysteine oxidation is one of the main causative mechanisms of cardiovascular damage.

Keywords: Hydrogen peroxide; Homocysteine; Chemiluminescence; Oxidants; Antioxidants

Abbreviations: CL, chemiluminescence; DTPA, diethylenetriaminepentaacetic acid; DHR, dihydrorhodamine; RH, rhodamine; SIN-1, 3-morpholinosydnonimine

INTRODUCTION

Homocysteine is a non protein forming amino acid, situated at the branch point of two metabolic pathways: transsulfuration to cystathionine and transmethylation to methionine.[1]

Blood concentration of homocysteine is in the micromolar range and moderately high levels are

*Corresponding author. Lab. di Chimica Clinica—Policlinico A. Gemelli, Largo A. Gemelli, 8-00168 Rome, Italy. Tel.: +39-06-30154222. Fax: +39-06-35501918. E-mail: b.zappacosta@uniserv.ccr.rm.cnr.it

considered an independent risk factor for atherosclerosis and for a variety of thrombotic complications.[2–5]

Although epidemiological evidence underlines a strong association between high plasma homocysteine concentration and vascular thrombotic diseases, the molecular mechanisms of its toxicity are not fully understood.[4] The toxicity of homocysteine is commonly attributed to different pathophysiological mechanisms such as activation of factors XII and V,[6,7] inhibition of C-protein activation,[8] impaired regulation of endothelium-derived relaxing factor,[9] stimulation of smooth muscle cell proliferation.[10] Most of the proposed mechanisms, however, are based on a common molecular process, possible oxidative damage, induced by homocysteine. Homocysteine, in fact, could exert a pro-oxidant action through the production of hydrogen peroxide (H_2O_2) during its metal-catalyzed oxidation.[11] Moreover, many authors have proposed a molecular mechanism for homocysteine-mediated damage involving free radical production.[12,13] In particular, superoxide anion ($O_2^{\cdot-}$), in presence of nitric oxide, can form the powerful oxidant peroxinitrite[14] at the same time decreasing the bioavailability of nitric oxide, a potent vasorelaxant.[15]

Unfortunately, the relevance of these hypotheses is greatly limited by the high concentrations of homocysteine used in these *in vitro* experiments, often in the millimolar range, higher than those observed in patients with inherited or acquired hyperhomocysteinemia.[9–11,16,17]

In a recent paper,[18] we have shown that homocysteine, even at physiological concentrations, significantly quenches the chemiluminescence produced by the oxidative burst of activated polymorphonuclear leukocytes, in contrast to what has been reported by Olinescu.[19] Since these data are not consistent with the reputed pro-oxidant action of homocysteine, we intend to determine accurately the homocysteine-dependent H_2O_2 production by using a very sensitive method[20] and explore the eventual pro-oxidant effects of homocysteine through interaction with highly oxidizing species like hypochlorite, peroxynitrite and ferrylmyoglobin, which are known to be involved in cardiovascular damage.[21]

MATERIALS AND METHODS

Chemicals

DL-homocysteine, horse radish peroxidase (HRP), H_2O_2, homocysteine, DTPA (diethylenetriaminepentaacetic acid), 3-morpholinosydnonimine (SIN-1), horse heart myoglobin (Mb) and luminol were from Sigma (St Louis, MO, USA); dihydrorhodamine (DHR) and Amplex Red were from Molecular Probes (Eugene, OR). All other reagents were of the highest purity available from commercial sources. The homocysteine standard solution (1 mM), was freshly prepared in 18 MΩ double-distilled deionized water (MilliQ, Millipore, Bedford, MA) and tested according to a modification of the HPLC method of Araki and Sako,[22] as already described.[5]

Chemiluminescence (CL) measurements were carried out on an automatic luminometer (Berthold LB953, Wildbad, Germany).

H_2O_2 Production

Amplex Red, a colorless and non fluorescent derivative of resorufin, can be oxidized by H_2O_2, in the presence of HRP, producing a highly fluorescent product. H_2O_2 production by homocysteine was then calculated by measuring the specific fluorescence of oxidized Amplex Red molecule (Ex = 350, Em = 399 nm) according to a modification of the method of Zhou.[20] The amount of H_2O_2 was measured against a standard H_2O_2 curve; in our system, the

minimum amount of H_2O_2 thus detectable was 15 pmoles.

The H_2O_2 production by homocysteine was obtained at 37°C in phosphate buffer (50 mM, pH 7.4) in the presence of 50 μM homocysteine; the incubation was carried out in the presence or absence of 100 μM DTPA in order to verify the metal-dependent H_2O_2 production or in the presence of 100 IU/ml catalase in order to confirm the H_2O_2-dependent Amplex Red oxidation. The measurements of H_2O_2 production were performed at 30 min intervals for 270 min; briefly, a 1 ml aliquot of each homocysteine preparation was added to a cuvette containing 2 ml of 1 μM Amplex Red, 1.2 IU/ml HRP and 150 μM DTPA, and the fluorescence intensity measured on a Fluoromax spectrofluorometer (Spex Industries, Edison, NJ, USA).

CL of the System HCY–luminol–ClO^-

Homocysteine (0–1000 μM), 50 μM luminol and 70 μM ClO^- (final concentrations) were added in 1.0 ml final volume of sodium-acetate buffer (0.1 M, pH = 5.1).

CL was started with the addition of 100 μl of ClO^- and expressed as integral counts for 5 s.

Dihydrorhodamine Oxidation

Oxidation of DHR (25 μM) to rhodamine (RH) by HClO (1–10 μM) was determined by measuring the increase in absorbance at 500 nm in the absence and presence of 10 μM homocysteine.

Interaction with Met and Ferrylmyoglobin

Ferrylmyoglobin (Mb^{IV}) formation was monitored in solutions (2 ml final volume) containing metmyoglobin (Mb^{III}) (50 μM heme) and homocysteine (100 μM). The interaction of Mb^{IV}, obtained by incubating metmyoglobin (50 μM) with 150 μM H_2O_2,[23] and homocysteine, was monitored at 550 nm. Catalase (500 units/ml) was added before the addition of homocysteine to remove residual H_2O_2.

Interaction with Peroxynitrite

Peroxynitrite ($ONOO^-$) was obtained by spontaneous decomposition of SIN-1 at neutral pH, and the peroxynitrite specific oxidation of DHR (50 μM) to RH at 500 nm in 50 mM phosphate buffer, 0.1 mM DTPA, pH 7.4, was followed spectroscopically at 500 nm at 37°C. Homocysteine at different concentrations was added 12 min after SIN-1, i.e. when the rate of $ONOO^-$ formation was constant.

Spectrophotometric Assays

All the spectrophotometric assays were performed on a Hewlett–Packard 8450A UV/Vis. spectrophotometer equipped with a cuvette stirring apparatus and a constant temperature cell holder.

Statistics

All the experiments were performed in triplicate. Results are expressed as means ± SE.

RESULTS

The production of hydrogen peroxide by 50 μM homocysteine, is shown in Fig. 1. In the presence of a chelating agent (DTPA), no production of H_2O_2 can be observed confirming a strict dependence on metal ions. Addition of 100 IU/ml catalase inhibited Amplex Red oxidation, emphasizing that the oxidizing species is H_2O_2, produced by a metal-catalyzed oxidation of homocysteine. The rate of H_2O_2 production is 235 pmoles/min/μmoles homocysteine.

Figure 2 shows the CL response of the HClO–luminol system in the presence of different concentrations of homocysteine. CL is strongly

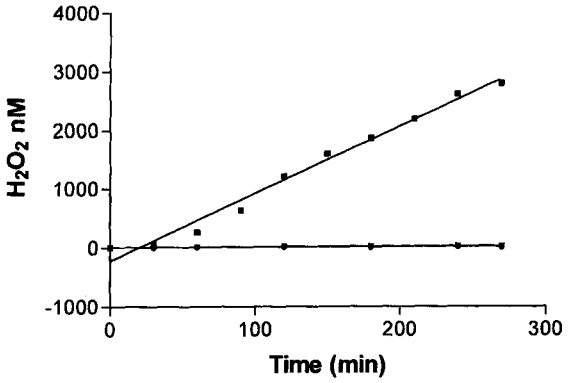

FIGURE 1 H_2O_2 production by homocysteine in presence (▼) or in absence (■) of DTPA, and with catalase (●).

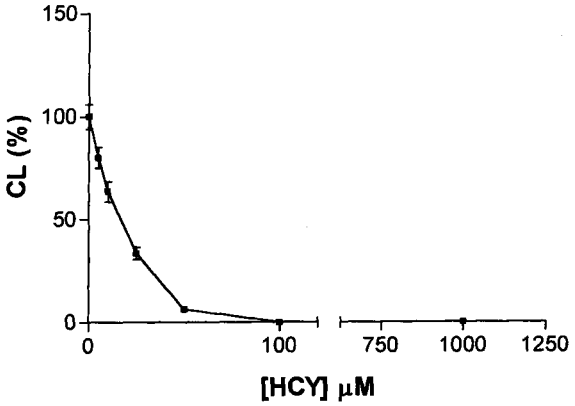

FIGURE 2 Effect of homocysteine on luminol–hypochlorite luminescence. Different concentrations of homocysteine were added to 50 μM luminol and 70 μM HClO in Na-acetate buffer 0.1 M, pH 5.1. Chemilumunescence started after addition of HClO and was expressed as integral count over 5 s.

inhibited by homocysteine even at very low concentration (IC_{50} is 17 ± 5 μM).

Figure 3 shows that the HClO-dependent oxidation of DHR to RH, a usually stoichiometric reaction, is completely inhibited by 10 μM homocysteine.

Both Figs. 2 and 3 clearly indicate that homocysteine is an efficient scavenger of HClO.

Peroxynitrite is the product of the nearly diffusion-limited reaction between superoxide anion ($O_2^{\cdot-}$) and nitric oxide (NO). Peroxynitrite is a powerful oxidant that damages many cellular components[14] and experimental probes such as DHR.[24] As shown in Fig. 4, the oxidation of DHR by peroxynitrite (dotted line) is a time-dependent process that approaches zeroth-order kinetics after ~10 min. The addition of homocysteine (solid line) at concentrations corresponding to physiological levels slightly inhibits DHR oxidation. At higher levels of homocysteine (1 mM) a strong protection of DHR against $ONOO^-$ oxidation can be observed.

Moreover, the effect of homocysteine on ferrylmyoglobin, a highly reactive species, was investigated. Figure 5a shows the spectroscopic transitions from Mb^{III} (absorption peaks at 502, 582 and 632 nm) to Mb^{IV} (absorption peaks at 546 and 586 nm) induced by the oxidation afforded by H_2O_2 and the back conversion of Mb^{IV} to Mb^{III} in the presence of 100 μM homocysteine. When Mb^{III} is incubated with homocysteine in place of

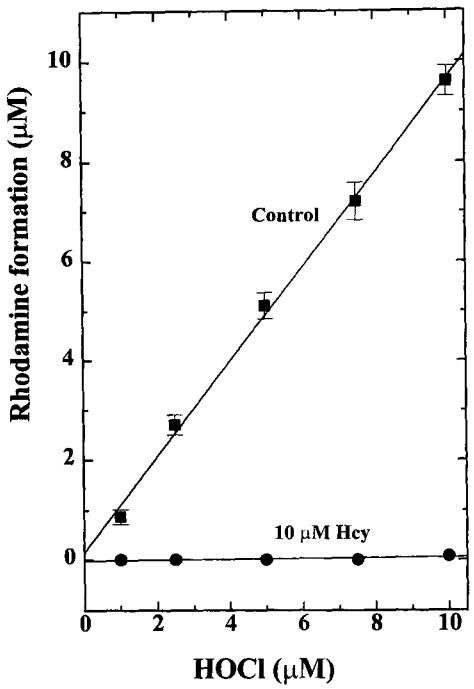

FIGURE 3 Effect of homocysteine on hypochlorite-dependent oxidation of dihydrorhodamine. Rhodamine formation was determined by measuring the absorbance at 500 nm in the absence (■) and presence (●) of 10 μM homocysteine.

150 μM H_2O_2, Mb^{IV} is not formed at all. The rate of conversion of Mb^{IV} to Mb^{III} in the presence of different concentrations of homocysteine is shown in Fig. 5b.

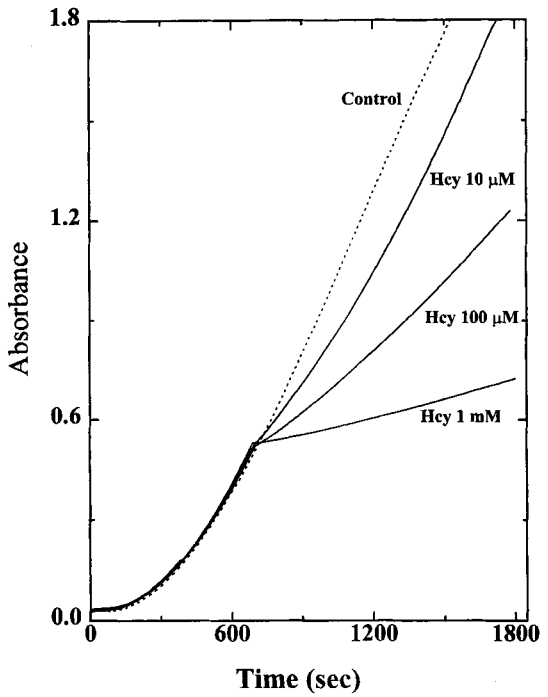

FIGURE 4 Kinetics of dihydrorhodamine oxidation by peroxynitrite in absence (.....) or in presence (—) of different homocysteine concentrations.

DISCUSSION

Hyperhomocysteinemia was related to cardiovascular disease basically on epidemiological grounds. Notwithstanding, few *in vitro* studies have focused on the mechanism of this correlation.

Starkerbaum[11] showed a toxic effect of homocysteine on cultured endothelial cells due to hydrogen peroxide generation induced through copper-catalyzed homocysteine oxidation. In agreement with these data, other authors demonstrated that homocysteine promotes lipid peroxidation in presence of redox active transition metals.[25]

Consistent with these experimental findings, many studies have claimed that increased blood homocysteine concentration could induce the endothelial cell damage through a free radical mediated oxidative mechanism.[11] Unfortunately most of these *in vitro* experiments have been performed using unreal *in vivo* concentrations of homocysteine (in millimolar range).

However, some authors,[26] although confirming a significant correlation between moderate hyperhomocysteinemia and cardiovascular disease, have also argued whether homocysteine, *per se*, is responsible for the cardiovascular damage, mainly because its blood concentration is in the micromolar range and, therefore, well under the level that can generate H_2O_2 in significant amount.

The results of this paper clearly show that homocysteine produces a negligible quantity of H_2O_2 (only 1:4000 mole H_2O_2/mole HCY) and only when catalyst metal ions are present. It is important to note that the level of H_2O_2 measured in our system is almost 20 times higher than the sensitivity of the method used (15 pmoles). To the best of our knowledge, no paper has yet reported an assessment of H_2O_2 production by homocysteine with such a sensitive and precise method.

Our results seem to support the above criticisms,[25] indicating that in our *in vitro* systems homocysteine does not behave as a pro-oxidant.

As a matter of fact, we have found that homocysteine, besides quenching luminol-dependent CL of activated polymorphonuclear leukocytes,[18] strongly inhibits the hypochlorite induced oxidation of both luminol and dihydrorhodamine to aminophtalate and rhodamine, respectively. This effect is particularly interesting since polymorphonuclear leukocytes produce HClO during their activation.[27]

Furthermore, homocysteine does not enhance peroxynitrite formation but conversely inhibits DHR oxidation by $ONOO^-$ (see Fig. 4). Nonetheless, homocysteine does not oxidize metmyoglobin to ferrylmyoglobin, but, on the contrary, reduces Mb^{IV} to Mb^{III} (see again Fig. 5).

All these results clearly indicate that homocysteine at micromolar concentrations, i.e. at concentrations usually found in normal and

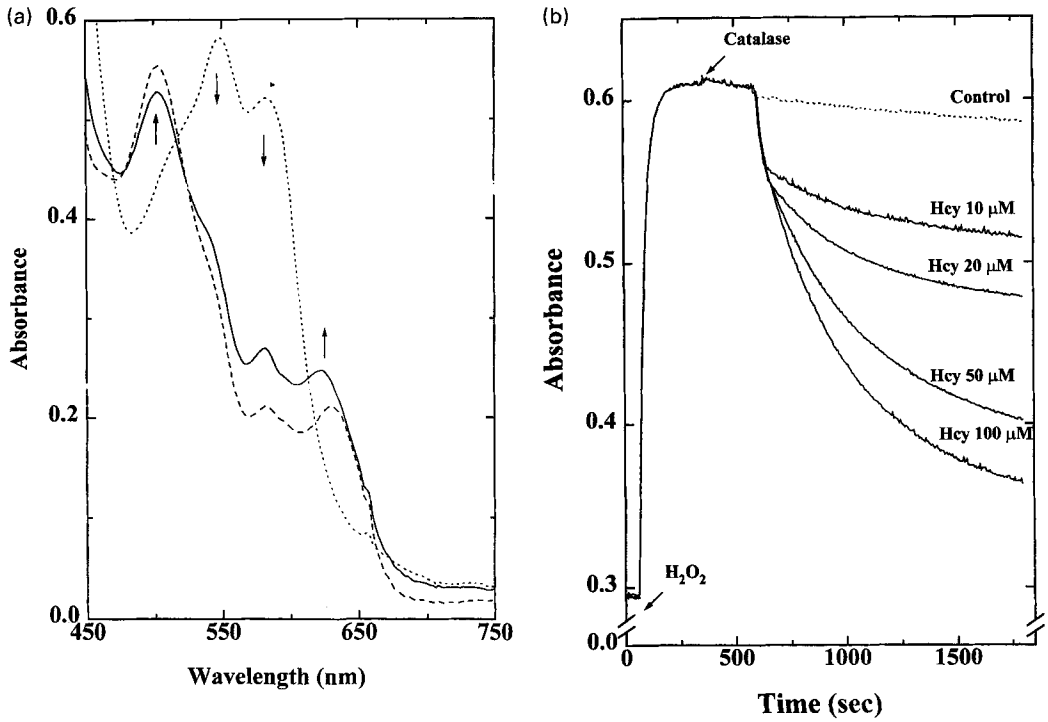

FIGURE 5 (a) Reduction of myoglobin^{IV} by homocysteine: spectroscopic changes of myoglobin^{III} (---) to myoglobin^{IV} (.....) after reaction with H_2O_2 and its back-conversion to myoglobin^{III} (—). (b) Homocysteine-dependent reduction of Mb^{IV}. The reduction of Mb^{IV} to Mb^{III} by different homocysteine concentrations was monitored as decrease in absorbance at 550 nm.

vasculopathic subjects (5–50 μM), does not act as a pro-oxidant but, on the contrary, displays an antioxidant effect both on cellular[18] and chemical systems.

Therefore, as should be expected from thiols (glutathione or cysteine) or other redox compounds (ascorbic acid or α-tocoferol) in absence of decompartimentalized metal catalysts (low molecular weight metals[28]), homocysteine seems to exert a protective effect against highly stressing/oxidizing molecules rather than to behave as a straightforward pro-oxidant.

In view of this, the increase of homocysteine concentration correlated with vasculopathic disorders could be interpreted as a reactive (perhaps even protective) condition resulting from,[29] or concurring with, the oxidative stress associated with vascular damage more than a causative factor.

References

[1] Mudd, S.H., Levy, L.H. and Skobvy, F. (1995) "Disorders on transsulfuration", In: Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., eds, The Metabolic Basis of Inherited Disease, 6th ed. (Mcgraw Hill, New York), pp 1279–1327.

[2] Ueland, P.M. and Refsum, H. (1989) "Plasma homocysteine, a risk factor for vascular disease: plasma levels in health, disease and drug therapy", *Journal of Laboratory and Clinical Medicine* **81**, 2004–2006.

[3] Malinow, M.R. (1994) "Homocyst(e)ine and arterial occlusive diseases", *Journal Internal Medicine* **17**, 236–603.

[4] D'Angelo, A. and Selhub, J. (1997) "Homocysteine and thrombotic disease", *Blood* **90**, 1–11.

[5] De Stefano, V., Zappacosta, B., Persichilli, S., Rossi, E., Casorelli, I., Paciaroni, K., Chiusolo, P., Leone, A.M., Giardina, B. and Leone, G. (1999) "Prevalence of mild hyperhomocysteinemia and association with thrombofilic genotypes (Factor V Leiden and prothrombin G20210A) in Italian patients with venous thromboembolic disease", *British Journal Haematology* **106**, 564–568.

[6] Ratnoff, O.D. (1968) "Activation of Hageman factor by L-homocysteine", *Science* **162**, 1007–1009.

[7] Rodgers, G.M. and Kane, W.H. (1986) "Activation of endogenous factor V by homocysteine-induced vascular endothelial cell activator", *Journal Clinical Investigation* **77**, 1909–1916.

[8] Rodgers, G.M. and Conn, M.T. (1990) "Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells", *Blood* **75**, 895–901.
[9] Stamler, J.S., Osborne, J.A., Jaraki, O., Rabbani, L.E., Mullins, M., Singel, D. and Loscalzo, J. (1993) "Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen", *Journal Clinical Investigation* **91**, 308–318.
[10] Tsai, J.C., Parrella, M.A., Yoshizumi, M., *et al.* (1994) "Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis", *Proceedings of the National Academy of Sciences of the USA* **91**, 6369–6373.
[11] Starkebaum, G. and Harlan, J.M. (1986) "Endothelial cell injury due copper-catalyzed hydrogen peroxide generation from homocysteine", *Journal Clinical Investigation* **77**, 1370–1376.
[12] Loscalzo, J. (1996) "The oxidant stress of hyperhomocysteinemia", *Journal Clinical Investigation* **98**, 5–7.
[13] Olszewski, A.J. and McCully, K.S. (1993) "Homocysteine metabolism and the oxidative modification of proteins and lipids", *Free Radicals in Biology and Medicine* **14**, 683–693.
[14] Pryor, W.A. and Squadrito, G.L. (1995) "The chemistry of peroxinitrite: a product from the reaction of nitric oxide with superoxide", *American Journal Physiology* **268**, L699–L722.
[15] Crow, J.P. and Beckman, J.S. (1995) In: Ignarro, L. and Murad, F., eds, Nitric Oxide: Biochemistry, Molecular Biology and Therapeutic Implications (Academic, CA), pp. 17–43.
[16] Dudman, N.P.B., Hicks, C., Wang, J. and Wilcken, D.E.L. (1991) "Human arterial endothelial cell detachment *in vitro*: its promotion by homocysteine and cysteine", *Atherosclerosis* **91**, 77–83.
[17] Zhang, F., Slungaard, A., Vercellotti, G.M. and Iadecola, G. (1998) "Superoxide-dependent cerebrovascular effects of homocysteine", *American Journal Physiology* **43**, R1704–R1711.
[18] Zappacosta, B., Mordente, A., Persichilli, S., Giardina, B. and De Sole, P. (2000) "Effect of homocysteine on polymorphonuclear leukocytes activity and luminol-dependent chemiluminescence", *Luminescence* **16**, 165–168.
[19] Olinescu, R., Kummerow, F.A., Handler, B. and Fleischer, L. (1996) "The haemolytic activity of homocysteine is increased by the activated polymorphonuclear leukocytes", *Biochemical and Biophysical Research Communications* **226**, 912–916.
[20] Zhou, M., Diwu, Z., Panchuk-Voloshina, N. and Haugland, R.P. (1995) "A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases", *Analytical Biochemistry* **253**, 162–168.
[21] Giulivi, C. and Cadenas, E. (1994) "Ferrylmyoglobin: Formation and chemical reactivity toward electron donating compounds", *Methods in Enzymology* **133**, 404–409.
[22] Araki, A. and Sako, Y. (1987) "Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detector", *Journal of Chromatography* **422**, 43–52.
[23] Galaris, D., Cadenas, E. and Hochstein, P. (1989) "Redox cycling of myoglobin and ascorbate: a potential protective mechanism against oxidative reperfusion injury in muscle", *Archives Biochemistry and Biophysics* **273**, 497–504.
[24] Crow, J.P., Beckman, J.S. and McCord, J.M. (1995) "Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxinitrite", *Biochemistry* **34**, 3544–3552.
[25] Heinecke, J.W., Kawamura, M., Suzuki, L. and Chait, A. (1993) "Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms", *Journal of Lipid Research* **34**, 2051–2061.
[26] Green, R. (1998) "Homocysteine and occlusive vascular disease: culprit or bystander?", *Preventive Cardiology* **3**, 31–33.
[27] Klebanoff, S.J. and Clark, R.C. (1978) The Neutrophil: Function and Clinical Disordes (Amsterdam, North-Holland).
[28] Cavallini, D., Denmarco, C., Duprè, S. and Rotilio, G. (1969) "The copper catalyzed oxidation of cysteine to cystine", *Archives Biochemistry and Biophysics* **130**, 354–361.
[29] Dudman, N.P.B. (1999) "An alternative view of homocysteine", *Lancet* **354**, 2072–2074.