In Vitro Interaction Between Homocysteine and Copper Ions: Potential Redox Implications

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Homocysteine (Hcys) has been implicated in various oxidative stress–related disorders. The presence of a thiol on its structure allows Hcys to exert a double-edge redox action. Depending on whether Cu^{2+} ions occur concomitantly, Hcys can either promote or prevent free radical generation and its consequences. We have addressed *in vitro* the interaction between Hcys and Cu^{2+} ions, in terms of the consequences that such interaction may have on the free radical scavenging properties of Hcys and on the redox state and redox activity of the metal. To this end, we investigated the free radical–scavenging, $O_2^{\cdot -}$-generating, and ascorbate-oxidizing properties of the interacting species by assessing the bleaching of $ABTS^{\cdot +}$ radicals, the reduction of $O_2^{\cdot -}$-dependent cytochrome *c*, and the copper-dependent oxidation of ascorbate, respectively. In addition, electron paramagnetic resonance and Cu(I)-bathocuproine formation were applied to assess the formation of paramagnetic complexes and the metal redox state. Upon a brief incubation, the Hcys/Cu^{2+} interaction led to a decrease in the free radical–scavenging properties of Hcys, and to a comparable loss of the thiol density. Both effects were partial and were not modified by increasing the incubation time, despite the presence of Cu^{2+} excess. Depending on the molar Hcys:Cu^{2+} ratio, the interaction resulted in the formation of mixtures that appear to contain time-stable and ascorbate-reducible Cu(II) complexes (for ratios up to 2:1), and ascorbate- and oxygen-redox–inactive Cu(I) complexes (for ratios up to 4:1). Increasing the interaction ratio beyond 4:1 was associated with the sudden appearance of an $O_2^{\cdot -}$-generating activity. The data indicate that depending on the molar ratio of interaction, Hcys and Cu^{2+} react to form copper complexes that can promote either antioxidant or pro-oxidant actions. We speculate that the redox activity arising from a large molar Hcys excess may partially underlie the association between hyperhomocysteinemia and a greater risk of developing oxidative-related cardiovascular diseases. Exp Biol Med 231:1569–1575, 2006

Key words: homocysteine; copper ions; free radicals; antioxidants; complex formation; redox consequences

Introduction

Homocysteine (Hcys), the demethylated derivative of methionine, is a major precursor of cysteine (Cys) for the synthesis of glutathione (1). The occurrence of a thiol moiety on its structure endows Hcys with the ability to transfer a hydrogen atom to free radical species, thereby contributing to the overall antioxidant status (2). In addition, the thiol group confers Hcys the ability to donate an electron to an oxidizing substrate, thus behaving as a biological reductant (2, 3). In the presence of a transition metal such as copper, Hcys has the potential to reduce Cu^{2+} into the redox active Cu^{+} ion (3). The latter species rapidly catalyzes the reduction of molecular oxygen into a superoxide anion, and that of hydrogen peroxide into a hydroxyl radical (4, 5). Consequently, Hcys also has the potential to behave as an endogenous pro-oxidant. The oxidative stress arising from a copper dependent free radical generation may underlie, in part, the correlation existing between hyperhomocysteinemia and a higher risk of developing certain oxidative stress related cardiovascular (6–8) and neurodegenerative (9, 10) diseases. Vast evidence supports the contention that hyperhomocysteinemia represents an independent risk factor for atherosclerosis (11, 12); thus, the current working hypothesis implicates the auto-oxidation of Hcys as a cause of the increased formation of reactive oxygen species (ROS) that underlie both the oxidative stress and the damage affecting endothelial cells (4, 13, 14). In addition, some experimental evidence implicates a reduced availability of nitric oxide in the development of the endothelial dysfunction and atherosclerosis associated with hyperhomocysteinemia (15).

Copper excess (16, 17), by favoring free radical generation, and copper deficiency (18), by resulting in an

This work was supported by FONDECYT (grant 1040736).

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1535-3702/06/2319-1569$15.00

inadequate function of copper dependent antioxidant mechanisms, both have been implicated as factors leading to an increased risk of developing oxidative stress related vascular disorders. Thus, copper ions are abundantly present in mature atherosclerotic lesions (19) and are most effective in inducing low-density lipoprotein (LDL) oxidation in experimental models (20); copper levels are also increased in diseased coronary arteries compared with healthy coronary arteries (21). In agreement with the latter, epidemiological data show a positive association between plasma copper levels and the risk of developing cardiovascular diseases (22, 23). On the basis of the aforementioned evidence and on the potential of Hcys to function either as a pro-oxidant or as an antioxidant molecule (or both), several studies have addressed the effects that the interaction between Hcys and copper may have on the oxidation of various biological targets and on the oxidative stress related cell dysfunction. Presumably, such interaction implies a reduction of Cu^{2+} ions by Hcys, which followed by an increased production of ROS (4, 13, 24), results in an enhancement of the toxicity exerted by either copper or Hcys alone. Thus, early work by Starkebaum and Harlan (4) showed that Hcys plus copper dose-dependently induce the lysis of cultured human and bovine endothelial cells. Subsequently, Hcys was shown to synergistically increase the oxidative stress and the cytotoxicity induced by copper ions in HeLa and in endothelial ECV304 cells (13), to induce apoptosis in the latter when associated with Cu^{2+} ions (25), and to potentiate copper-mediated toxicity in cultured neurons (26).

In contrast with such observations, other *in vitro* studies, conducted largely in target macromolecules, indicate that the interaction between copper and Hcys would not necessarily lead to or potentiate oxidative damage. For instance, Halvorsen *et al*. (24) reported that whereas low concentrations of Hcys enhance Cu^{2+}-induced oxidation of LDL, high concentrations of this amino acid protect this particle, delaying the onset of oxidation of its lipids and proteins as long as thiol groups of Hcys remain available. Likewise, other investigators (27, 28) coincide in reporting that Hcys protects LDL against copper-induced damage rather than promoting its oxidation, and that it fails to increase the cleavage of DNA molecules induced by copper ions (29). Prompted by this apparent copper dependent double-edge role of Hcys; namely, its antioxidant versus pro-oxidant effects, in the present study we have addressed *in vitro* the interaction between Hcys and Cu^{2+} ions, in terms of the consequences that such interaction may exert on the free radical-scavenging properties of the thiol–amino acid and on the redox state and redox activity of the metal.

Materials and Methods

Chemicals and Reagents. 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), cupric chloride ($CuCl_2 \cdot 2H_2O$), cytochrome *c* (Cyt *c*; bovine heart), Hcys, L-ascorbic acid (AA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), L(-)histidine (His), and bathocuproinedisulfonic acid disodium salt (BATO) were all purchased from Sigma, St. Louis, MO. 2,2′-Azo-bis (2-amidinopropane dihydrochloride) (AAPH) was obtained from Waco Pure Chemicals Industries Ltd. Osaka, Japan. Unless indicated otherwise, all solutions employed in the present studies were prepared in Chelex-100–treated sodium phosphate buffer (10 m*M*; pH 7.4), and all experiments were conducted at 22°C.

Bleaching Capacity Assay. The stable chromoactive free radical $ABTS^{\bullet+}$ was generated by incubating (45°C, 55 mins) ABTS (75 μ*M*) with the thermo-unstable azo-derivative AAPH (2 m*M*). AAPH generates peroxyl radicals, which upon interaction with ABTS, give place to a green and stable colored $ABTS^{\bullet+}$ solution (OD_{734nm}). The OD_{734nm} of the colored solution remained constant for at least 4 hours (22°C). Changes in the OD_{734nm} resulting from the addition of Hcys alone (4 μ*M*), or a preincubated (for 3 or 30 mins) mixture of Hcys plus Cu^{2+}, were monitored spectrophotometrically (Unicam Helios-α, Cambridge, England). The addition of Cu^{2+} or the copper chelator, His, to a cuvette containing $ABTS^{\bullet+}$ had no effect on the OD_{734nm} (30). The bleaching capacity was estimated and expressed as the difference in optical density (OD) of an $ABTS^{\bullet+}$-containing solution seen at Times 0 and 3 mins after the addition of Hcys (ΔOD_{734nm}).

Thiol Content Determination. Solutions of Hcys (20 μ*M*), or mixtures of this thiol plus Cu^{2+} (preincubated for 3 or 30 mins), were incubated with DTNB (0.3 m*M*) in buffer containing His (1 m*M*). His was used to prevent Cu^{2+} from catalyzing the reoxidation of thio-nitrobenzoic acid (TNB) (generated during the titration), and was found not to affect the ability of Hcys/Cu^{2+} to bleach $ABTS^{\bullet+}$. The OD_{412nm} of the resulting solutions was read against blanks containing no thiol.

Cu(I)-Bathocuproine Assay. In the presence of a reductant such as ascorbate, BATO binds copper ions, giving place to a stable Cu(I)-$(BATO)_2$ complex (Cu(I)-BATO). In the absence of ascorbate, the thiol-mediated formation of such a complex was assessed by monitoring the increase in OD_{480nm} that follows the addition of a mixture of Hcys with Cu^{2+} (20 μ*M*), preincubated for 3 and 30 mins, onto a BATO (360 μ*M*) containing solution.

Copper-Dependent Ascorbate Oxidation Assay. Ascorbate oxidation was assessed by measuring the loss of OD_{265nm} that follows the addition of Cu^{2+} (20 μ*M*) to an ascorbate (50 μ*M*) solution. To assess the effect of Hcys on Cu^{2+}-induced ascorbate oxidation, a mixture of this thiol plus Cu^{2+} was preincubated for 3 and 30 mins and added to the ascorbate solution.

Cytochrome *c* Reduction Assay. The superoxide-dependent reduction of Cyt *c* was assessed by monitoring the increase in OD_{550nm} that follows the addition of a mixture of Cu^{2+} (10 μ*M*) plus Hcys to a solution containing Cyt *c* (50 μ*M*).

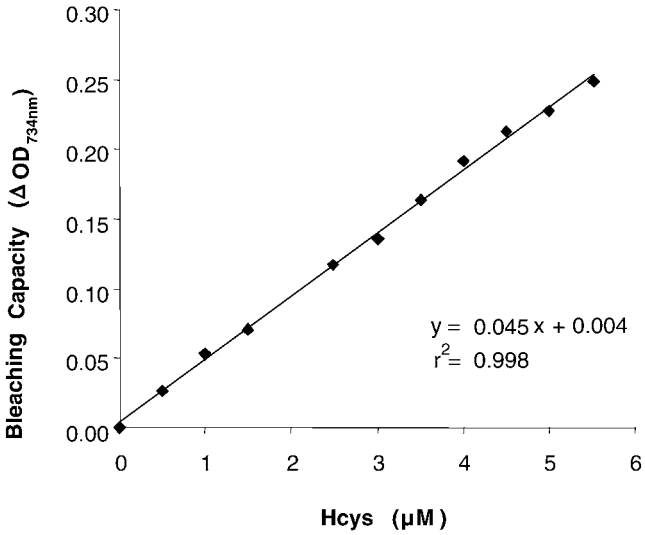

Figure 1. Concentration dependence of the bleaching of $ABTS^{\cdot+}$ radicals by homocysteine. Increasing concentrations of Hcys were added to a solution containing $ABTS^{\cdot+}$. The bleaching (i.e., a drop in the OD_{734nm}) was measured 2 mins after the addition of the thiol (longer times, up to 10 mins, gave identical results).

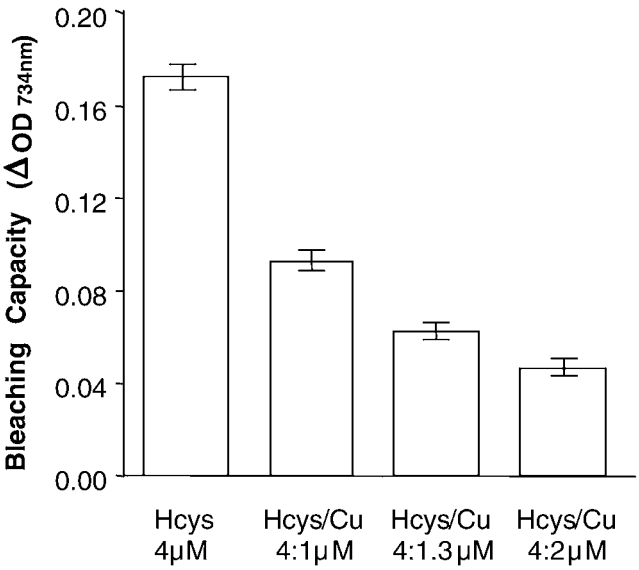

Figure 2. Effects of copper ions on the bleaching capacity of homocysteine. The $ABTS^{\cdot+}$ bleaching capacity of a preincubated mixture (during 3 mins) of Hcys (4 μ*M*) plus Cu^{2+} (1, 1.3, or 2 μ*M*) was assayed at OD_{734nm} as described in the Materials and Methods section. The bleaching (i.e., a drop in the OD_{734nm}) was measured 2 mins after the addition of these mixtures (monitoring for longer times, up to 10 mins, gave identical results). Experiments were conducted in triplicate. Results are presented as mean ± SEM. Differences between bars were statistically different, with $P < 0.05$.

Electron Paramagnetic Resonance Studies. To explore the possible formation of paramagnetic complexes between Hcys and Cu^{2+} ions (5 m*M*), the effect of adding increasing concentrations of Hcys on the Cu^{2+} electron paramagnetic resonance (EPR)–dependent signal was studied using EPR. Spectra were recorded in a Bruker ECS 106 spectrometer, using an X band (9.85 GHz), a rectangular cavity, and 50 KHz field modulation at 22°C under the following conditions: frequency, 9.79 GHz; center field, 3180 gauss; amplitude modulation, 9 gauss; microwave power, 25 W; time constant, 20 secs; time scan, 40 secs.

Results

Free Radical–Scavenging Capacity of Hcys: Effects of Cu^{2+} Ions. Figure 1 shows the ability of Hcys to bleach $ABTS^{\cdot+}$ radicals in a linear and concentration dependent manner. The preincubation of a fixed Hcys concentration (4 μ*M*) with increasing concentrations of Cu^{2+} (1, 1.3, or 2 μ*M*) led to a copper dependent decrease of the bleaching capacity (Fig. 2). Such effect was maximal for an Hcys:Cu^{2+} molar ratio of 4:2, and involved decreasing the bleaching capacity of Hcys to about one-third of that displayed by the thiol in the absence of copper. Identical results were found for Hcys/Cu^{2+} mixtures preincubated for 3 or 30 mins. Increasing the concentration of Cu^{2+} ions from 2 to 24 μ*M* while maintaining the concentration of Hcys at 4 μ*M* yielded identical results (not shown).

Thiol Density of Homocysteine: Effects of Cu^{2+}. To assess whether the decrease in the bleaching capacity that results from the interaction between Hcys and Cu^{2+} leads to a Cu-dependent change in the thiol density of mixtures, the reactivity of the latter toward DTNB was measured. As shown in Figure 3, the preincubation of a fixed concentration of Hcys with increasing concentrations of copper led to a marked decrease in the thiol titrateable groups. Such effect was maximal at a molar ratio of 20:6.6, and meant lowering the thiol density to approximately 40% of the control values. Identical thiol recoveries were found for thiol/Cu^{2+} mixtures preincubated for 3 or 30 mins.

Cu(I)-Bathocuproine Formation. The partial re-

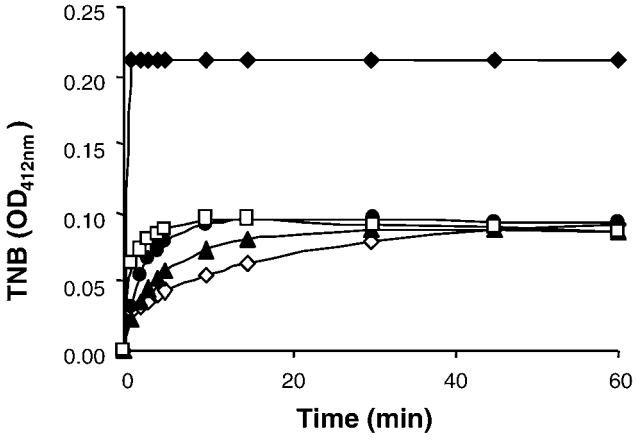

Figure 3. Effects of copper ions on the thiol content of Hcys plus copper mixtures. Hcys alone (20 μ*M* ♦) and mixtures of Hcys (20 μ*M*) plus increasing concentrations of Cu^{2+} (6.6 μ*M* ◇; 10 μ*M* ▲; 20 μ*M* ●; 60 μ*M* □) were preincubated (3 mins) and assayed for their thiol content. The latter was assayed as the reactivity toward DTNB displayed by the mixtures (along 60 mins), and it was expressed as the optic density at 412 nm of thio-nitrobenzoic acid (TNB).

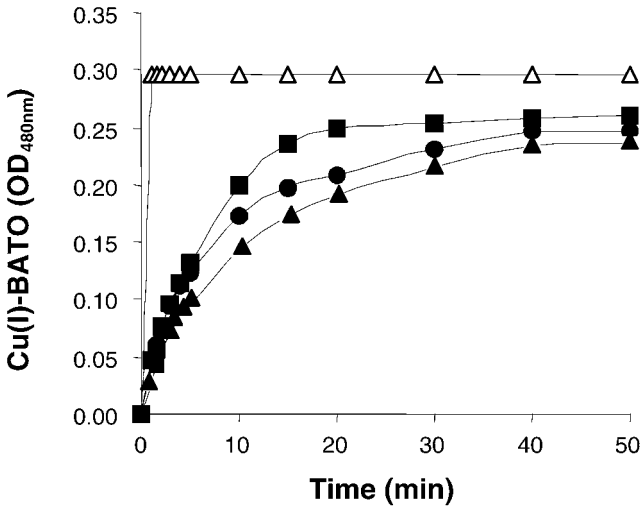

Figure 4. Effects of Hcys on Cu(I): BATO complex formation. Cu^{2+} (20 μM △) in the absence of the thiol (but in the presence of AA as a reductant), and mixtures of Cu^{2+} (20 μM) plus increasing concentrations of Hcys (20 μM ■; 60 μM ● and 80 μM ▲) were preincubated (3 mins) and added to a solution containing BATO (360 μM). The resulting change in optic density was measured at OD_{480nm}.

covery of thiols described above suggests that during the preincubation of Hcys and Cu^{2+}, cuprous ions are being formed. To assess the latter, the formation of a Cu(I)-BATO complex in the preincubated Hcys/Cu^{2+} mixtures was measured (these experiments were conducted in the absence of ascorbate as reductant). As shown in Figure 4, following the preincubation of Cu^{2+} ions with increasing concentrations of Hcys, most of the added copper, nearly 80%, was recovered as Cu(I)-BATO. Maximal Cu(I)-BATO formation was already seen for Hcys/Cu^{2+} mixtures with a molar ratio of 20:20, and (as seen above) identical results were obtained with mixtures preincubated for 3 or 30 mins. Although the maximal concentration of Cu(I)-BATO was the same for all the studied ratios, the rate at which it was reached was slower as the concentration of Hcys in the mixtures was increased.

EPR Studies on Hcys/Cu^{2+} Mixtures. Compared with a typical EPR spectrum of Cu^{2+} ions (Fig. 5A), mixtures of Hcys/Cu^{2+} (preincubated for 3 or 30 mins in molar ratios equal or lower than 10:5) resulted in the formation of an EPR signal (Fig. 5B) that is typically associated with the occurrence of tetrahedric or planar paramagnetic Cu(II) complexes (31). Such a paramagnetic spectrum was no longer observed when the mixtures were incubated in ratios equal to or greater than 15:5 (not shown).

Effects of Hcys on the Cu^{2+}-Dependent Oxidation of Ascorbate. *In vitro*, Cu^{2+} ions increase the rate of ascorbate oxidation (32) in a concentration-dependent manner. To understand the redox properties of the Cu(II) complexes putatively formed during the preincubation referred to above, the effect of Hcys on the ability of Cu^{2+} to catalyze the oxidation of AA was studied. The preincubation of a fixed concentration of Cu^{2+} with increasing concentrations of Hcys led to the loss of its ability to induce AA oxidation (Fig. 6). When preincubated at equimolar concentrations, Hcys was already able to prevent the oxidation of AA by about 30%; upon doubling the Hcys/Cu^{2+} molar ratio (40:20), the protection rose to about 90%, and was complete for ratios equal to or greater than 60:20. This effect of adding Hcys was identical whether the Hcys/Cu^{2+} mixtures were preincubated for 3 or 30 mins.

Effects of Hcys on the Reactivity of Copper Toward Oxygen. Based on the Cu^{2+} reducing capacity of Hcys, and on the ability of Cu^{+} ions to generate superoxide radicals during their interaction with oxygen, the ability of Hcys to either promote or inhibit $O_2^{\cdot -}$-dependent Cyt *c* reduction was evaluated. The direct addition of Hcys to a solution containing Cyt *c* and Cu^{2+} led to a rapid and substantial reduction of the cytochrome

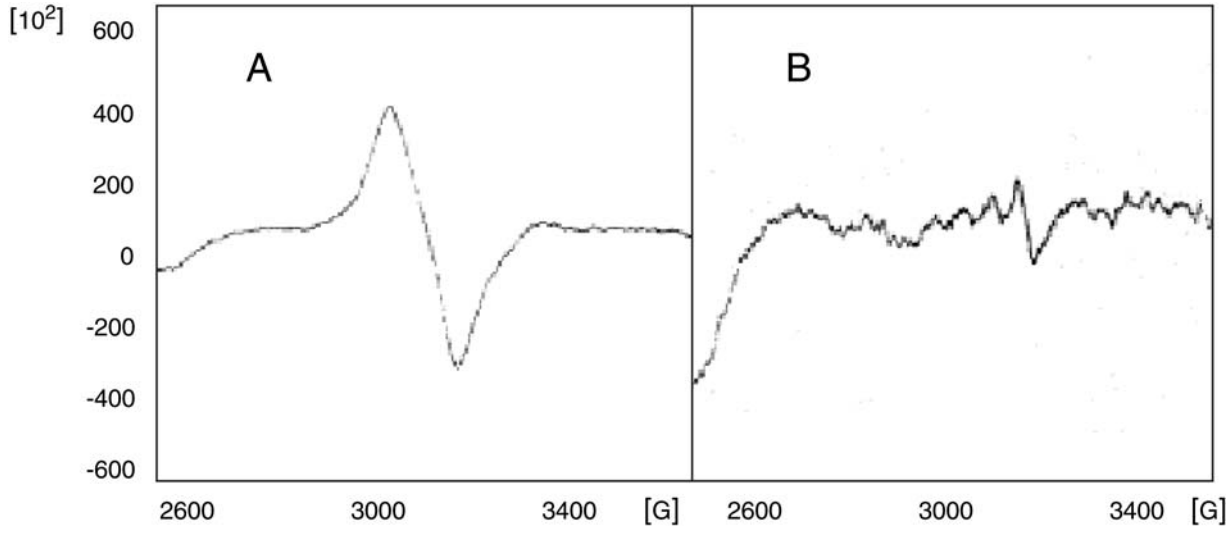

Figure 5. EPR spectra of Cu^{2+} ions in the absence or presence of Hcys. (A) Typical EPR spectrum of Cu^{2+} (5 mM). (B) Spectrum resulting from the incubation (for 3 mins) of a Cu^{2+} solution in the presence of Hcys (5mM).

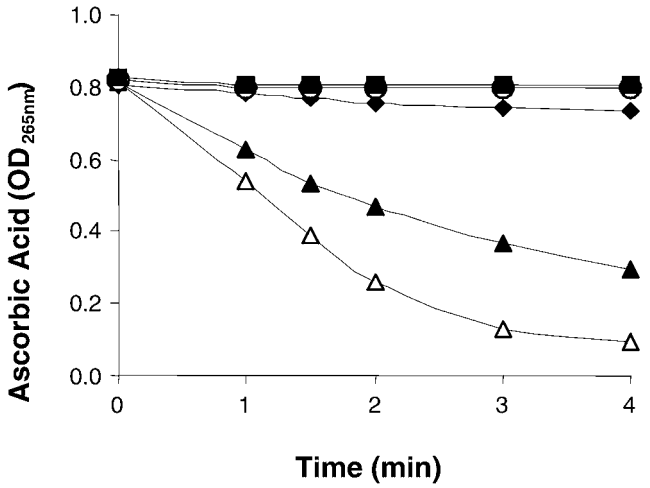

Figure 6. Effects of Hcys on Cu^{2+}-dependent ascorbate oxidation. Cu^{2+} (20 μM △) in the absence of the thiol, or mixtures of Cu^{2+} (20 μM) plus increasing concentrations of Hcys (20 μM ▲; 40 μM ◆; 60 μM ○, and 80 μM ■) were preincubated (during 3 mins) and added to a solution containing AA (50 μM). The resulting change in optic density was measured at OD_{265nm}.

(monitored for 2 mins; Fig. 7). In contrast, this effect was entirely absent when Hcys and Cu^{2+} ions were added as mixtures preincubated in a 10:10, 20:10, or 40:10 molar ratio (only the 40:10 results are presented in Fig. 7). Furthermore, we observed no reduction in Cyt *c* when a preincubated 40:10 mixture was added to a solution containing Cyt *c* and 40 μM Hcys (not shown). Starting from a molar ratio of 60:10, mixtures of Hcys/Cu^{2+} were found to be active in producing $O_2^{\bullet -}$ as evidenced by their ability to reduce Cyt *c*. We observed no differences in the

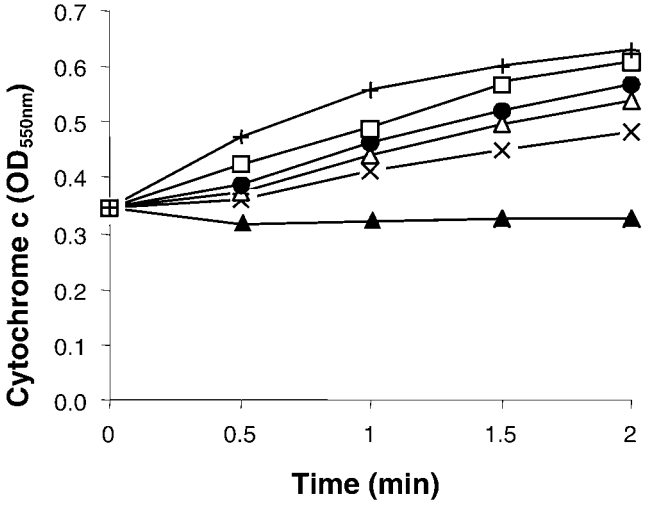

Figure 7. Effects of Hcys on Cu^{2+} mediated superoxide dependent Cyt *c* reduction. Preincubated mixtures (3 mins) of Hcys/Cu^{2+} (40:10 μM ▲; 60:10 μM ×; 80:10 μM △; 100:10 μM ●; 140:10 μM □), or nonpreincubated Hcys (40 μM) plus Cu^{2+} (10 μM) (+) were added to a solution containing Cyt *c* (50 μM). Superoxide dependent (SOD inhibitable) reduction of Cyt *c* was assessed by monitoring the rate of increase in OD_{550nm}.

results when using Hcys/Cu^{2+} mixtures preincubated for either 1 or 3 mins.

Discussion

The ABTS assay represents a simple and direct *in vitro* method to assess the free radical scavenging properties of a variety of hydrogen donor compounds (30, 33). Here, we used this methodology to investigate the ability of Hcys to bleach $ABTS^{\bullet +}$ radicals, and to assess the effects that the interaction between Hcys and Cu^{2+} ions has on the free radical scavenging properties of the thiol. Results indicate that at near-physiological plasma concentrations, Hcys bleaches $ABTS^{\bullet +}$ radicals, and that upon its incubation with Cu^{2+} ions, the latter concentration dependently diminish the scavenging capacity of the thiol. The maximal inhibitory effect of copper, which was attained at a Hcys:Cu^{2+} ratio of 4:2, suggests diminishing the scavenging activity of Hcys to only one-third of that displayed by the thiol in the absence of Cu^{2+}. Thereafter, neither the presence of a large copper excess, nor prolonging by 10-fold the preincubation time, affected the mixture's scavenging activity. On the basis of these observations, we suggest that Cu^{2+} ions interact with Hcys to form one or more complexes that would retain, at least partially, the free radical scavenging properties of the free amino acid. In line with such interpretation, we observed that incrementing the concentration of Hcys and Cu^{2+}, while keeping both species at a fixed ratio of 1:3 (namely, in the presence of Cu^{2+} excess), led to proportional increments in the bleaching capacity of the mixtures (not shown). Presumably, such increments indicate that proportionally greater concentrations of such putative complexes would form and become available to interact with $ABTS^{\bullet +}$ radicals. The proposed formation of such complexes is in line with a recent work by Apostolova *et al.* (34) showing that isolatable copper-homocysteine complexes are easily formed when mixing large concentrations of Hcys and Cu^{2+}. While the identity of the complexes formed under the present *in vitro* conditions remains to be established, our thiol titrating experiments suggest that such moieties are likely to be associated with the ability of the mixtures to bleach $ABTS^{\bullet +}$. Thus, approximately 40% of the thiols initially present in Hcys/Cu^{2+} mixtures were recovered as DTNB titrateable groups. Because the latter remained constant despite a large molar copper excess and regardless of the preincubation time, we suggest that the Hcys/Cu^{2+} interaction leads to the formation of complexes whose thiol content would account for the $ABTS^{\bullet +}$ scavenging activity of the mixtures. In fact, Hcys as such, or following its preincubation with Cu^{2+} ions, was entirely devoid of $ABTS^{\bullet +}$ bleaching capacity (not shown).

The partial recovery of thiols referred to above can be interpreted as an indication that a sizeable part of the added Hcys undergoes oxidation in a reaction involving the reduction of Cu^{2+}. In fact, most (around 80%) of the copper present in the Hcys/Cu^{2+} mixtures was recovered as a Cu(I)-

BATO. Because identical results were observed after 3 or 30 mins of preincubation of the mixtures, it would appear that upon formation, the reduced copper ions remain as such along the preincubation conditions. We suggest that such copper would not occur as free Cu^{+} ions but as Cu(I) coordinately bound to Hcys, forming complexes. The EPR studies reveal that at a molar ratio of 1:1, the interaction between Hcys and Cu^{2+} ions leads not only to the disappearance of the typical Cu^{2+} EPR spectrum, but to the appearance of a paramagnetic spectral signal, which is typically associated with the presence of Cu(II)-bound complexes (31). In the presence of a molar excess of Hcys (namely, ratios greater than 2:1) the EPR signal described above was found to disappeared completely, suggesting that Cu(I) containing complexes, expected to be EPR-silent, have been formed. Worth mentioning is that mixtures containing Cu(II) as well as Cu(I) complexes seem to be stable in time, because prolonging their preincubation (by even 10-fold) did not lead to the reappearance of any free Cu^{2+} EPR signal, nor did it result in any fading of the signal associated with the copper containing complexes. An experimental approach that lends indirect but further support to the occurrence of the postulated complexes in the Hcys/Cu^{2+} mixtures was provided by the oxidation ascorbate assay. Thus, while the oxidation of ascorbate resulting after the addition of mixtures containing low molar ratios (e.g., 1:1) suggest the presence of "reducible" Cu(II) complexes in such mixtures, the total absence of such oxidation observed after addition of high molar ratios mixtures (e.g., greater than 2:1) is in line with the presence of Cu(I) complexes in the latter. On the other hand, because prolonging by 10-fold the preincubation of mixtures containing Cu(I) complexes triggered no oxidation of ascorbate, it would appear that during the preincubation, Hcys swiftly stabilizes copper ions under a Cu(I) form, which would be redox inactive toward oxygen, such that no Cu^{+}/Cu^{2+} recycling occurs. Consistent with such a view, only the preincubated mixtures of Hcys/Cu^{2+} (up to 4:1), expected to contain Cu(I) complexes, failed to catalyze the $O_2^{\cdot -}$ dependent reduction of Cyt *c*. Most interesting, however, such redox inactivity was suddenly lost when the thiol:Cu^{2+} molar ratio was increased to 6:1. Further increments in the molar ratio of such mixture led to higher rates of $O_2^{\cdot -}$ generation, with a maximal rates of Cyt *c* reduction been attained with a molar ratio of 14:1. Thus, under such molar excess of Hcys, the thiol seems to "paradoxically" loss its capacity to form redox inactive complexes. We speculate that further understanding the latter might contribute to explain the association between hyperhomocysteinemia and a greater risk of developing oxidative related cardiovascular damage.

On the other hand, the formation of redox-inactive Cu(I) complexes could be seen as a mechanism not only to decrease the ability of copper ions to catalyze free radical generation, but also to prevent the premature and indiscriminate binding of copper ions to other sensitive macromolecules. Thus, Hcys-dependent stabilization of copper ions under the proposed complex forms might help to explain the reported failure of Cu^{2+} to induce, in the presence of Hcys, oxidative damage to either LDL (27, 28) or DNA (29). The authors of the present study certainly agree that cell toxicity can be induced under *in vitro* conditions by mixtures of Cu^{2+} plus Hcys (4, 13, 14, 24), and that the potential for such type of damage also exists *in vivo*. However, on the basis of *in vitro* data presented here, the possibility should be considered that the actual pro-oxidant (Cu^{2+}-reducing) properties of Hcys may be well counteracted by its potential to act as an antioxidant.

In summary, the present *in vitro* studies indicate that the interaction between Hcys and Cu^{2+} ions would lead to the formation of complexes that contain copper and thiol that are able to promote either antioxidant or pro-oxidant actions. Such complexes would partially retain the free radical scavenging properties displayed by the thiol alone, even in the presence of a large molar excess of Cu^{2+} ions. The interaction between equimolar concentrations of Hcys and Cu^{2+} ions appears to result primarily in time-stable and ascorbate reducible Cu(II) containing complexes. Under a low molar excess of Hcys, such interaction would, in turn, result in the formation of Cu(I) containing complexes that are time-stable, and ascorbate- and oxygen redox–inactive (for ratios up to 4:1). We observed, however, that further increasing the ratio of Hcys:Cu^{2+} interaction (beyond 4:1), was associated with the swift appearance of a substantial reactivity toward oxygen ($O_2^{\cdot -}$ generating). We propose that to the extent to which the *in vivo* conditions allow Hcys and Cu^{2+} ions to interact, the molar ratio at which both species can react would be the key in defining the overall biological effects associated with their interaction.

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