Inhibitory Effect of Histamine H₃-Receptor Antagonist Cimetidine against Copper(II)/Ascorbate- mediated Protein Damage

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The inhibition of ascorbate-mediated protein damage by the histamine H₂-receptor antagonist, cimetidine, was chemically studied. In the presence of copper(II) ion, ascorbate gave rise to oxidative modification of both a protein and a histidine derivative under simulated physiological conditions (pH 7.2, 37°C), whereas the reactions were entirely inhibited by the addition of cimetidine. The inhibition of protein damage resulted in almost total disappearance of cimetidine within 24 hr and the appearance of two major products, a species (2) assigned as the 2-imidazolone derivative of cimetidine and cimetidine sulfoxide (3). The mechanism for the protective effect of cimetidine against copper(II)/ascorbate-mediated protein damage is discussed.

In the presence of a catalytic amount of metal ions, ascorbate can trigger a chain reaction and generate highly reactive intermediates of molecular oxygen.¹⁻⁴⁾ The function of ascorbate is to reduce the metal ion ($M(n+1)$; Eq. (1)), and to serve as a source for superoxide (O_2^- ; equation 2) and hydrogen peroxide (H_2O_2 ; equation 3). The reduced metal ion [$M(n)$] is conducted *via* a Fenton reaction to generate the most potent oxidant, the hydroxyl radical ($\cdot OH$; Eq. (4)).

These partially reduced oxygen species have been implicated by many authors to be important causative agents of oxygen toxicity in cancer, aging, several human diseases, and food deterioration. These intermediates may be responsible for the action of various biological and synthetic materials such as enzymes, antibiotics, carcinogens, and re-

ducing materials like ascorbate under physiological conditions.^{5,6)}

In vitro, a metal/ascorbate system promotes the oxidative scission of various food and biological materials such as polysaccharides,⁷⁾ proteins,⁸⁻¹²⁾ and DNA.¹³⁻¹⁵⁾ Especially, it has been suggested that the active free radical species generated by the metal-catalyzed systems tend to react with the first available target in the ligand (protein), resulting in specific damage rather than random injury to the protein.^{11,16)} In relation to this, we have confirmed that Cu(II)/ascorbate reacts preferentially with histidine residues in proteins¹²⁾ and peptides.¹⁷⁾

Recently, we have found that some nitrogenous compounds inhibited such ascorbate-mediated protein damage. Among the compounds tested, cimetidine was regarded as protecting the protein best of all. Cimetidine, marketed under the trade name Tagamet, is a potent histamine H₂-receptor antagonist, which inhibits excessive acid secretion caused by histamine,^{18,19)} and is widely used clinically for treating of peptic ulcers.

This study was embarked upon to investigate the influence of imidazole-related

materials on ascorbate-dependent protein damage and to discover a novel antioxidant against free radical damage. In this study, we demonstrate a unique property of cimetidine not only as a chelating agent for Cu(II) ion but as an efficient inhibitor against free radical damage. We also describe the characterization of the major oxidation products of cimetidine, including an unknown compound.

Materials and Methods

Materials. Cimetidine and *N*-benzoylhistidine were obtained from the Sigma Chemical Company (St. Louis, MO) and bovine serum albumin from Seikagaku Kogyo Co., Ltd. (Tokyo). Cimetidine sulfoxide was obtained from Simth Kline Fujisawa Co., Ltd. (Tokyo), and trifluoroacetic acid (>99%) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). All other reagents were of the highest grade commercially available.

Reaction conditions. The protective effect of cimetidine was assayed by monitoring its ability to inhibit the Cu(II)/ascorbate-mediated damage of bovine serum albumin or a histidine residue analogue (*N*-benzoylhistidine). The reactions were performed in a 2 ml reaction mixture (0.1 M phosphate buffer, pH 7.2) containing 0.04% bovine serum albumin or 1 mM *N*-benzoylhistidine, 5 mM ascorbate and 0.05 mM CuSO₄ in the presence and absence of cimetidine. The reaction mixtures were stirred for an additional 30 min at 37°C. The reactions were initiated by the addition of ascorbate and stopped by the addition of EDTA (0.1 M).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Protein damage during incubation with Cu(II)/ascorbate was monitored by SDS-PAGE of samples of the reaction mixture. The method of Laemmli²⁰ was used with running gels containing 12% acrylamide. For this, the sample proteins were collected by precipitating with 12% trichloroacetic acid. The gel was run at 20 mA for 2 hr, stained in 0.25% Coomassie Brilliant Blue R-250 in water-2-propanol-acetic acid (5:5:1, v/v/v), and destained in 7% acetic acid containing 10% methanol.

High performance liquid chromatography (HPLC). Protein damage was also chromatographically determined by gel filtration on a TSK-GEL G3000 SW column (7.5 × 600 mm). Samples were eluted with 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl at a rate of 1.0 ml/min, the elution being monitored by absorbance at 210 nm. Loss of *N*-benzoylhistidine was determined by HPLC Reverse-phase chromatography, using a Develosil ODS-5 column (4.6 × 250 mm). The samples were eluted

with a solution of 25% methanol in 0.1% trifluoroacetic acid at a rate of 0.8 ml/min, the elutions being monitored by absorbance at 210 nm. Loss of cimetidine and the concomitant formation of the products were also determined by HPLC on a Develosil ODS-5 column under the same HPLC conditions as those for *N*-benzoylhistidine. Areas of the chromatographic peaks for each material were calculated by the use of a Shimadzu Chromatopac integrator C-R3A.

Isolation of the oxidation product of cimetidine. The reaction was performed in 6 ml of phosphate buffer (pH 7.2) containing 10 mM cimetidine, 50 mM ascorbate, and 0.5 mM CuSO₄. The reaction mixture was kept at 37°C for 24 hr. After terminating the reaction with EDTA (1 mM), the mixture was freeze-dried and then subjected to preparative HPLC on a Develosil ODS-10 column (24 × 300 mm). The product was eluted with a solution of 20% methanol in 0.1% trifluoroacetic acid at a rate of 5 ml/min, the elutions being monitored by absorbance at 210 nm. The eluate was fractionated and further purified by using a Develosil ODS-5 column.

Instrumental analyses. The nuclear magnetic resonance (NMR) spectrum on a JEOL JNM-FX200 spectrometer was taken in dimethylsulfoxide-*d*₆ with tetramethylsilane as the internal standard. Fast atom bombardment-mass spectrometry (FAB-MS) was performed with a JEOL JMS-DX705 mass spectrometer, for which the samples were dissolved in dimethylsulfoxide and added to a drop of glycerol on the target.

Results and Discussion

Figure 1 demonstrates the SDS-PAGE patterns of bovine serum albumin treated with Cu(II)/ascorbate in the presence and absence of cimetidine. Incubation of bovine serum albumin with Cu(II)/ascorbate gave rise to significant changes of the protein band in the absence of cimetidine, while the ascorbate-dependent protein damage was inhibited by the addition of cimetidine in a dose-dependent manner. It is apparent from the results that protein damage was completely inhibited by adding cimetidine above 0.5 mM. In addition, it is of particular interest to note that the inhibition of protein damage resulted in the almost total disappearance of cimetidine by itself within 24 hr (Fig. 2). These results suggest that the active species generated by Cu(II)/ascorbate reacted with cimetidine more preferentially than the protein, which resulted

Fig. 1. Protective Effect of Cimetidine against Oxidative Damage to Bovine Serum Albumin Mediated by Copper (II)/Ascorbate.

Solutions containing 0.04% bovine serum albumin, 0.05 mM Cu(II) and 5 mM ascorbate in 0.1 M phosphate buffer (pH 7.2) were incubated for 8 hr at 37°C in the presence (lanes c–g) and absence (b) of cimetidine. Lane a represents native bovine serum albumin. The concentrations of cimetidine were 0 mM (lane b), 0.05 mM (lane c), 0.1 mM (lane d), 0.5 mM (lane e), 1.0 mM (lane f), and 10 mM (lane g).

in the selective loss of cimetidine.

On the other hand, we have previously shown that Cu(II)/ascorbate gave rise to selective damage to the histidine residues in protein, which resulted in the oxidative modification of protein by itself.¹²⁾ Hence, we examined the protective effect of cimetidine against the loss of the histidine residues in protein by using a histidine analogue (*N*-benzoylhistidine). As shown in Fig. 3, the histidine derivative was also protected by cimetidine and, along with the protection of the histidine derivative, cimetidine almost disappeared concomitantly in the reaction. It is apparent that this result is quite analogous to that in Fig. 2. Moreover, the loss of cimetidine in the Cu(II)/ascorbate system was greatly accelerated in an O₂ atmosphere, while it was completely retarded in an N₂ atmosphere (data not shown). The reaction was therefore confirmed to be O₂-dependent, suggesting the participation of active oxygen species. Consequently, these results suggest

Fig. 2. Inhibition of Protein Damage and Concomitant Loss of Cimetidine.

Solutions of 0.04% bovine serum albumin (○—○), 1 mM cimetidine (△—△) and 0.04% bovine serum albumin + 1 mM cimetidine (●—●, ▲—▲) were exposed to the Cu(II)/ascorbate system composed of 5 mM ascorbate and 0.05 mM Cu(II) ion. Symbols: circle, protein (bovine serum albumin); triangle, cimetidine. The protein and cimetidine were determined by HPLC on gel-filtration and reversed-phased columns, respectively.

Fig. 3. Inhibition of Histidine Damage and Concomitant Loss of Cimetidine.

Solutions of 1 mM *N*-benzoylhistidine (○—○), 1 mM cimetidine (△—△) and 1 mM *N*-benzoylhistidine + 1 mM cimetidine (●—●, ▲—▲) were exposed to the Cu(II)/ascorbate system composed of 5 mM ascorbate and 0.05 mM Cu(II) ion. Symbols: circle, *N*-benzoylhistidine; triangle, cimetidine. The histidine derivative and cimetidine were determined by HPLC on a reversed-phase column.

that cimetidine reacted directly with the active species generated by Cu(II)/ascorbate, which resulted in the protection of protein and the histidine derivative against oxidative damage. Hence, we attempted to characterize the oxidized products of cimetidine.

Fig. 4. HPLC Profile of the Copper (II)/Ascorbate-mediated Oxidation of Cimetidine.

A reaction mixture composed of 1 mM cimetidine, 5 mM ascorbate and 0.05 mM Cu(II) in phosphate buffer (pH 7.2) was incubated for 24 hr at 37°C. Peak 1 represents cimetidine and the others (2 and 3) represent reaction products.

As shown in Fig. 4, the reaction of cimetidine with Cu(II)/ascorbate gave two major products, the yields of **2** and **3** based on the substrate being 35% and 30%, respectively. Structural assignments to the products were made on the basis of their spectral properties and by a direct comparison with authentic samples on HPLC chromatograms. In order to characterize the chemical structure of **2**, isolation by preparative HPLC on reversed-phase columns was undertaken. Subsequently, its structural elucidation was performed by FAB-MS, ^1H -NMR and ^{13}C -NMR spectrometry. The spectral data of **2** were as follows: FAB-MS m/z 269 ($M+1$), 291 ($M+\text{Na}$); ^1H -NMR δ (ppm)=9.57 (1H, s), 9.49 (1H, s), 7.00 (2H), 3.41 (2H, s), 3.26 (2H, m), 2.67 (3H, s), 2.51 (2H, t), 1.88 (3H, s); ^{13}C -NMR δ (ppm)=159.5, 154.6, 117.9, 114.4, 112.2, 40.7, 29.9, 28.1, 25.8, 9.7. The spectral data of cimetidine were as follows: FAB-MS m/z 253 ($M+1$); ^1H -NMR δ (ppm)=7.50 (1H, s), 7.18 (1H, s), 3.66 (2H, s), 3.34 (2H, m), 2.73 (3H, s), 2.61 (2H, t),

Fig. 5. Structures of Cimetidine (**1**), the 2-Imidazolone Derivative of Cimetidine (**2**) and Cimetidine Sulfoxide (**3**).

2.14 (3H, s); ^{13}C -NMR δ (ppm)=159.6, 133.1, 130.0, 124.8, 117.9, 40.7, 29.8, 28.1, 25.8, 9.6. The quasimolecular ion having m/z 269 in **2** corresponds to the monooxygenated form of cimetidine. In addition, the spectral data for ^1H -NMR and ^{13}C -NMR apparently suggest an alteration of the imidazole moiety of cimetidine. The ^1H -NMR spectrum of **2** exhibits the disappearance of the C-2 proton signal at 7.50 ppm in the imidazole group and the appearance of new proton signals at 9.57 and 9.49 ppm, corresponding to amide protons. The ^{13}C -NMR spectrum exhibits a shift of olefinic carbons at 130.0 ppm and 124.8 ppm to 117.9 ppm and 114.4 ppm and, in addition, a new signal at 154.6 ppm, which is characteristic in the ureido carbonyl group ($-\text{NHCO}-\text{NH}-$),²¹⁻²³ can be observed. Consequently, **2** was elucidated to be the 2-imidazolone derivative of cimetidine (Fig. 5). On the other hand, **3** was found to be identical to authentic cimetidine sulfoxide by HPLC. Taking into account the formation of products **2** and **3**, the loss of cimetidine by Cu(II)/ascorbate may have been due mainly to oxidation of the imidazole and sulfide moieties of cimetidine.

The potent antioxidative activity of cimetidine against the Cu(II)/ascorbate-mediated protein damage may be interpreted by the unique chelation ability of cimetidine with the Cu(II) ion. Cimetidine is known to be

a potential chelating agent that binds the Cu(II) ion.²⁴⁾ Recently, the Cu(II) ion has been demonstrated to dramatically increase the binding of cimetidine to the imidazole receptors located in rat brain.^{25,26)} The Cu(II) ion has been implicated in the regulation of the cimetidine binding sites in the brain. Of particular interest is the fact that the addition of ascorbate or dithiothreitol further enhances cimetidine binding.²⁷⁾ In relation to this, Kimura *et al.* have found that the Cu(II) ion tightly bound to other materials such as serum albumin or amino acids was rapidly released to bind with cimetidine in the presence of ascorbate.²⁸⁾ Considering our results, the Cu(I) ion bound to cimetidine may have been conducted to free radical reactions, which resulted in the oxidation of the ligand (cimetidine) by itself to generate oxidation products **2** and **3**. In relation to this, we have found that cimetidine reacted with $\cdot\text{OH}$ generated by γ -irradiation of water, Fe(II)/H₂O₂ (Fenton's reagent), and Fe(II)/EDTA/ascorbate (Udenfriend's reagent) more effectively than with the well-known $\cdot\text{OH}$ scavengers such as mannitol and dimethyl sulfoxide (Uchida *et al.*, unpublished results). Therefore, we speculate that cimetidine functions not only as a specific chelating agent

for Cu(II) but as an excellent free radical scavenger. The ligand exchange reaction of cimetidine with a Cu(II) complex and the subsequent oxidation of cimetidine mediated by ascorbate are summarized in Fig. 6.

On the other hand, Cu(II)/ascorbate-mediated protein damage can be inhibited by other chelating agents. EDTA also completely inhibits Cu(II)/ascorbate-mediated protein damage with chelation of the copper ion; in this case, the copper ion is completely inactivated and, therefore, ascorbate autooxidation does not occur. However, cimetidine binds the copper ion without inactivation of the metal ion, and ascorbate is oxidized with a copper-cimetidine complex to generate an active species which attacks a ligand (cimetidine) by itself. Therefore, the mechanism for inhibition by cimetidine against Cu(II)/ascorbate-mediated protein damage is unique and quite different from that by EDTA or other chelators.

The product distribution from *in vitro* oxidation of cimetidine has been well established.^{29,30)} Various sulfoxidation and *N*-demethylation products have been isolated from the reaction of cimetidine with Fenton's reagent [Fe(II)/H₂O₂] as a biomimetic model for mixed-function oxidase drug metabolism. The products from the reaction of cimetidine with Cu(II) and ascorbate may partly be consistent with those from the reaction with Fenton's reagent; however, the 2-imidazolone derivative of cimetidine (product **2**) has never been detected in Fenton's reaction.

As to the active species in Cu(II)/ascorbate, $\cdot\text{OH}$ has long been regarded as the major determinant for biological damage; however, we consider that a diffusible $\cdot\text{OH}$ may not be responsible for the formation of the 2-imidazolone compound (**2**). To determine whether the active species of this specific oxygenation was $\cdot\text{OH}$ or not, the reaction of cimetidine with Fenton's reagent [Fe(II)/H₂O₂] was examined; however, there was no 2-imidazolone product at all. In addition, the reaction of the substrate with Cu(II)/ascorbate was not prevented by $\cdot\text{OH}$ scavengers (data

Fig. 6. Proposed Mechanism for the Ligand Exchange Reaction of Cimetidine with a Cu(II)-Protein Complex and Subsequent Oxygenation of Cimetidine.

not shown). Therefore, $\cdot\text{OH}$ was excluded as the active species in the formation of product **2**.

Thus, the mechanism for the formation of product **2** in the Cu(II)/ascorbate system remains to be investigated. At the present stage, we speculate that the oxocopper(III) species generated by the reductive degradation of the copper-peroxo complex is the most probable oxidant to cimetidine. The process of ascorbate autoxidation may definitely involve electron-transfer from ascorbate to the copper(II) ion bound to cimetidine (Eq. (5)).²⁸⁾ The Cu(I) complex reacts subsequently with molecular oxygen to give an O_2 -adduct (Eq. (6)). The Cu(I)-O_2 complex is probably followed by the generation of the Cu(II)-O_2^- complex (Eq. (7)), and then the metal-peroxo complex $\text{Cu(II)-O}_2\text{H}$ degrades to the strongly oxidizing oxocopper(III) species (Eq. (8)).³¹⁾ The oxocopper(III) species has been postulated as the hydroxylating species in such mono-oxygenases as tyrosinase and dopamine β -hydroxylase.³³⁾

A subsequent reaction mechanism for oxygenation of the C-2 position of the imidazole ring with the oxocopper(III) intermediate remains unclear; however, we speculate that the formation of an azaoxometallacyclic intermediate, which has been postulated in the *ortho* hydroxylation of the pyridine nucleus with copper(II)/iodosylbenzene,³²⁾ is the most probable route for the formation of the 2-imidazolone structure.³¹⁾ The oxocopper(III) species is presumed to oxidize the sulfide moiety of cimetidine. However, it is difficult to rule out the participation of other oxidants such as H_2O_2 , since it has been established that H_2O_2 can easily react with the sulfide moiety of cimetidine to generate cimetidine sulf-

oxide (3).²⁹⁾

From our study, it became apparent that the formation of the free radicals gave rise to specific oxidation of imidazole and the sulfide moieties of cimetidine when the free radical reactions were initiated by ascorbate with the Cu(II) bound to cimetidine. This study has been focused on the antioxidative activity of cimetidine against protein damage, while a further study concerning its antioxidative activity against oxidative damage to other foods and biological materials will be needed. In relation to this, a study on the scavenging activity of cimetidine against other active oxygen species like singlet oxygen ($^1\text{O}_2$) and $\cdot\text{OH}$ is now in progress.

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