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Dynamics of reactive oxygen species generation in the presence of copper(II)–histidine complex and cysteine

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Histidine–copper(II) complex (Cu-His_2) is a form of bound copper necessary for cellular copper uptake. Due to the high affinity of histidine to copper(II) ions, the binding of copper(II) by histidine is considered a substantial part of plasma antioxidative defense. Also cysteine plays a role in the antioxidative system. However, we show here that in the presence of oxygen the histidine–copper(II) complex plus cysteine produces reactive oxygen species (ROS). Cysteine concentration was assayed using a thiol specific silver-mercury electrode. Hydrogen peroxide was assayed amperometrically using platinum electrode. ROS formation was followed by chemiluminescence of luminol-fluoresceine-enhanced system. Addition of cysteine to Cu-His_2 solution at pH 7.4 in the presence of atmospheric oxygen initiates the synthesis of H_2O_2 and generation of ROS, which manifests as a burst of chemiluminescence. The reaction has two stages; in the first stage, cysteine is utilized for the synthesis of an unstable intermediary product which becomes a substrate for ROS formation. Anaerobic conditions inhibit ROS formation. Increased cysteine concentration enhances the lag phase of the oxidative burst without influencing the amount of ROS. The synthesis of ROS (measured by chemiluminescence) is proportional to the concentration of Cu-His_2 employed. ROS production can be repetitively initiated by further additions of cysteine to the reaction medium. The study suggests that Cu-His_2 catalyzes cysteine-dependent reduction of oxygen to superoxide employing an intermediary cysteine-copper(I) complex and enabling Fenton reaction with copper and hydrogen peroxide produced as a secondary product. In effect, Cu-His_2 with cysteine may be a source of ROS in biological media.

Key words: Cysteine copper complex, cysteine oxidation, hydrogen peroxide formation, histidine copper complex

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INTRODUCTION

Biological antioxidative defense systems integrate actions of a variety of low-molecular-mass compounds and enzymes that prevent the formation of major biological oxidants. Among the low-molecular-mass antioxidants, compounds with reducing properties (classified as free radical scavengers) play a crucial role. These include common metabolites such as uric acid, bilirubin, biliverdin, carotenoids, flavonoids, cysteine and glutathione. Another group of compounds participating in the antioxidative defense are proteins sequestering transient metal ions from biological fluids. The best known members of this group include ceruloplasmin (de Silva & Aust,1993) controlling Cu^{2+} ions, and ferritin, lactoferrin and transferrin chelating iron (Halliwell, 1994). Low molecular-mass-antioxidants and metal-binding proteins act concurrently in the body, protecting proteins and other compounds from damage by reactive oxygen species generated by cellular metabolism (Tubaro *et al.*, 1998).

Cysteine, whose concentration in the human plasma is about 250 μM, is a crucial component of the thiol antioxidant system in the extracellular space. It is the main constituent of a specific redox thiol-disulphide buffer which determines properties of numerous proteins and low molecular mass compounds (Biswas *et al.*, 2006). About 3–10% of the total plasma cysteine is present in a free, reduced form (Ueland, 1995; Giustarini *et al.*, 2011). The thiol group of cysteine participates in various scavenging reactions of free radicals, reduction of lipid peroxides, hydrogen peroxide, and in chelating ions of transient metals. Therefore cysteine, either free or as a component of proteins, is necessary for the prevention of Fenton reaction (Hegde *et al.*, 2010).

Ceruloplasmin binds the majority of Cu^{2+} maintaining body fluids free of copper ions, and a fraction of copper ions are also bound to free histidine (Sarkar & Kruck, 1967) and histidine residues of plasma albumin (Deschamps *et al.*, 2003; Moriya *et al.*, 2008). Moreover, some plasma copper ions also associate with cysteine (Sandstead, 1995), threonine, glutamine and asparagine (Casella & Gullotti, 1983; Brumas *et al.*, 1993; Deschamps *et al.*, 2005). As copper(I) is one of the most active substrates for Fenton reaction, ceruloplasmin and other copper chelators are important elements of the antioxidative defense system. Histidine is a component of the active centers of proteins responsible for the binding of transient metals (Cu^{2+}, $Fe^{2+/3+}$ and others) with high affinity. L-histidine also acts as a hydroxyl radical and singlet oxygen scavenger. Free histidine as well as carnosine and anserine (dipeptides containing histidine) bind copper ions to form high affinity complexes (Wade & Tucker, 1998; Hodak *et al.*, 2009) which participate in amino acid-dependent transmembrane transport of copper (Goode *et al.*, 1989). Free histidine and histidine incorporated into peptides and proteins is an essential component of the anti-oxidative defense system (Deschamps *et al.*, 2003; Mesu *et al.*, 2006). In the plasma and other body fluids histidine co-

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Abbreviations: Cu-His_2, histidine–copper(II) complex; RLU, Relative Light Units

exists with cysteine and other thiol compounds. However, the properties of the compound histidine–cysteine antioxidative system have not been studied so far. The purpose of the present study was to follow production of reactive oxygen species in a reaction medium combining cysteine and Cu-His_2 in the presence of atmospheric oxygen.

MATERIALS AND METHODS

The study of cysteine reaction with Cu-His_2 included analysis of varying cysteine and Cu-His_2 concentrations and their ratio on the generation of reactive oxygen species in phosphate buffer pH 7.4.

All reagents were of analytical grade. Working solutions were prepared using glass-distilled water (with resistivity of ≥ 18 MΩ/cm). Stock solution of L-cysteine (20 mM) (Sigma) was prepared in 55 mM sodium phosphate buffer, pH 7.4 (POCh Gliwice). Dilutions of the stock solution with the same buffer were prepared directly prior to use. Their final pH was adjusted to 7.4 with 0.5 M NaOH. The luminol-fluoresceine enhanced detection system was prepared from 5 mM luminol (Carl Roth) and 5 mM fluoresceine (Fluka). Solution of Cu-His_2 was obtained by mixing buffered solution of $CuCl_2$ (Sigma) and histidine (Sigma) in a molar ratio of 1:4. The two-fold histidine excess assures a lack of unbound copper ions.

The measurement of chemiluminescence was carried out every 1 or 5 s using a Lumat LB 9507 luminometr. The intensity of chemiluminescence was expressed in Relative Light Units (RLU) integrating the curve of chemiluminescence intensity (Drożdż *et al.*, 1998). Each measurement was performed in triplicate and the reported results are mean of the three values.

Measurement of reactive oxygen species generated in Cu-His_2 solution as a function of cysteine concentration

In a set of experiments samples of 10, 20, 30, 40, 50 µl of 10 mM cysteine solution in 55 mM phosphate buffer pH 7.4 were added to a reaction cell containing 500 µl of the luminol-fluoresceine enhanced detection system and 20 µl of Cu-His_2 (final Cu^{2+} concentration 0.1 mM) to give a final cysteine concentration from 0.2 to 1 mM. Chemiluminescence generated in this reaction was measured every 5 s. Data visualization was performed using Sigma Plot v.11.0 program.

In further experiments the basic experimental system was constituted with a modified concentrations ratio: 500 µl Cu-His_2, final Cu^{2+} concentration 0.1 mM, 20 µl luminol-fluoresceine enhanced detection system and 50 µl of 6 mM cysteine – final conc. 0.5 mM. This system was used as a reference (control) in the next experiments.

ROS formation under restricted access of oxygen was measured as described above but the reagents were first deaerated for 3 min by purging with nitrogen or helium (final O_2 concentration was not measured) and the reaction mixture in the measuring chamber was overlaid with liquid paraffin. All additional reagents added were pipetted under the paraffin layer using a Hamilton micro-syringe. The control included the same reagent system with free oxygen access.

To study the effect of cysteine on the Cu-His_2 absorption spectrum 500 µl of Cu-His_2 solution in 55 mM phosphate buffer pH 7.4 (final Cu^{2+} concentration 0.1 mM) and 50 µl of 6 mM cysteine were added to a 2.5-ml spectrophotometric quartz cuvette. The changes of the Cu-His_2 spectrum were recorded at 300 nm on a HELIOS γ spectrophotometer.

Measurement of cysteine concentration using electrochemical method

Cysteine concentration was determined using a silver mercury electrode and a reference Ag/AgCl electrode in saturated KCl solution by a procedure described earlier (Drożdż *et al.*, 2007). The analyses were carried out in a work cell (2.5 ml) filled up with 55 mM phosphate buffer pH 7.4 and a magnetic microstirring bar inside. After several minutes of incubation with working buffer to obtain electrochemical equilibrium between the buffer and the electrode, an electrovoltaic cell was produced by inserting a liquid junction connecting the work cell with the reference-electrode cell. The baseline electric potential (in mV) was measured for 3 to 30 min, using an Elmetron CP-401 millivoltmeter. Once the baseline potential was measured, cysteine solution of varying concentrations was added to make a reference cysteine standard curve. The final cysteine concentration was in the range of 0.01 to 20 mM. The electrochemical potential was stable within ± 2.0 mV.

Time-dependent cysteine concentration changes were also assessed when various cysteine amounts were added to the reaction medium containing Cu-His_2 in 55 mM phosphate buffer. These measurements were carried out following addition of 50 µl, 100 µl, 200 µl, 400 µl, 600 µl, 800 µl or 1000 µl aliquots of 10 mM cysteine solution into the work-cell containing 1200 µl of Cu-His_2 at 0.01 mM Cu^{2+}. The electrode potential was measured every 1 s until termination of the reaction. Measurement results were automatically re-calculated into cysteine concentration (mM) and expressed as a function of cysteine concentration versus reaction time.

Measurement of hydrogen peroxide formation by amperometry

H_2O_2 concentration was measured by amperometry employing a platinum electrode activated with *o*-phenylenediamine (o-PD) as described by Liu and Zweiler (2001). The electrode was activated by immersion in an *o*-phenylenediamine dihydrochloride buffer along with an auxiliary second platinum electrode and a silver/chloride reference electrode. The reference starting potential of the activated platinum electrode versus the silver/chloride electrode was +900 mV. The activated platinum electrode was washed with distilled water and stored in 55 mM phosphate buffer pH 7.4. The auxiliary platinum electrode was stored in distilled water.

The measurement of H_2O_2 was carried out in a work-cell containing 9 ml of phosphate buffer pH 7.4. The electrode starting potential versus the reference Ag/Cl^- electrode was 650 mV at room temperature. All measurements were performed in triplicate. The reference relationship between the H_2O_2 concentration and current was: H_2O_2 (µM) = 2.817 nA – 29.527. In order to measure H_2O_2 synthesis in the presence of cysteine, first 500 µl of 10 mM cysteine solution in 55mM phosphate buffer pH 7.4 was added to 9 ml of 55 mM phosphate buffer, incubated for 40 s (delay), then 400 µl of Cu-His_2 solution was added. If not stated otherwise, the final copper concentration employed was 0.1 mM. In serial measurements, consecutive aliquots of cysteine were added in 1-min intervals.

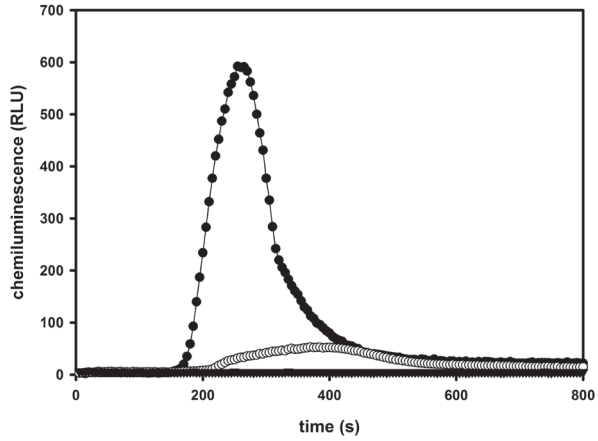

Figure 1. Generation of ROS in a reference system containing cysteine and Cu-His_2.
To 500 µl of Cu-His_2 in 55 mM phosphate buffer pH 7.4, (Cu^{2+} 0.1 mM) and 20 µl of luminol and fluoresceine (a chemiluminescence enhancer), 50 µl of 6 mM cysteine hydrochloride (●) was added to obtain final cysteine concentration 0.5 mM. Control sample (▲) contained the same reagents as above without cysteine. Another control (○) contained all the reagents except histidine. Chemiluminescence was measured at 5-s intervals.

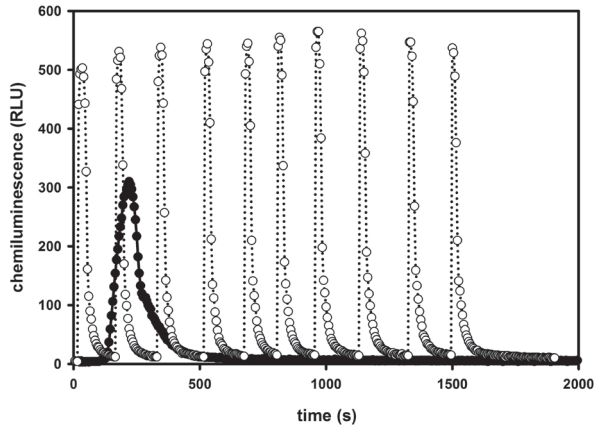

Figure 3. Effect of repeated additions of cysteine to the reaction medium with Cu-His_2.
Reaction conditions were as in Fig. 1, but cysteine was supplied in ten 5-µl aliquots of a 6 mM stock (total final concentration 0.5 mM), each added to the reaction mixture after termination of chemiluminescence (○). Chemiluminescence was measured at 5-s intervals. The reference system composed of cysteine — Cu-His_2 described in Fig. 1. was the control sample (●).

RESULTS

ROS synthesis in the cysteine — Cu-His_2 reaction system

Cysteine and Cu-His_2 in phosphate buffer pH 7.4 react and generate ROS, whose appearance is manifested as a chemiluminescence burst owing to the luminol-fluorsceine detection system. The reaction is specific for Cu-His_2 and does not occur without histidine as a complexing agent for copper ions. However, the addition of cysteine does not directly initiate the ROS synthesis since the burst of the chemiluminescence appears after some delay, the length of which depends on the final cysteine concentration (Fig. 1). Increasing the cysteine concentration from 200 to 1000 µM prolonged the delay of the light burst from 10 to 120 s (Fig. 2). The duration of the chemiluminescence increased proportionally to the cysteine concentration. At the highest cysteine concentration employed (1 mM) the light emission began at 120 s, achieved its maximum at 165 s, and terminated at about 200 s. The cysteine concentration also determined the intensity of the light emitted and the amount of ROS formed (calculated as the area under the RLU curve). However, with increasing cysteine concentration the amount of ROS formed per micromole of cysteine actually decreased (Table 1).

The amount of ROS generated depends not only on total amount of cysteine but also on dosing mode of the constant amount of thiol. When 50 µl of 6 mM cysteine was divided into two, five or ten portions and consecutively added to the Cu-His_2 solution after the end of the previous chemiluminescence burst, the amount of ROS formed (estimated as a sum of all oxidative bursts)

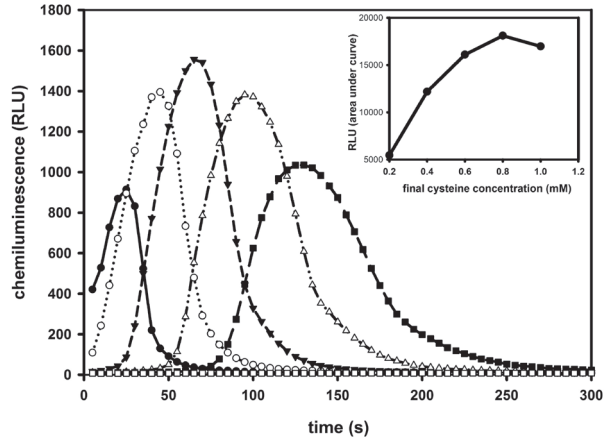

Figure 2. Effect of cysteine concentrations on ROS formation in the presence of Cu-His_2.
To 500 µl of luminol and fluoresceine (a chemiluminescence enhancer) and 20 µl of Cu-His_2 (final Cu^{2+} concentration 0.1 mM), different volumes of 10 mM cysteine were added (10–50 µl) to obtain final cysteine concentrations of 0.2 [●], 0.4 [○], 0.5 [▼], 0.7 [Δ], 9 [■] mM. Control sample [□] contained the same reagents as above without cysteine. Chemiluminescence was measured at 5-s intervals. The inset shows total of RLU intensity that corresponds to ROS generation as a function of cysteine concentration.

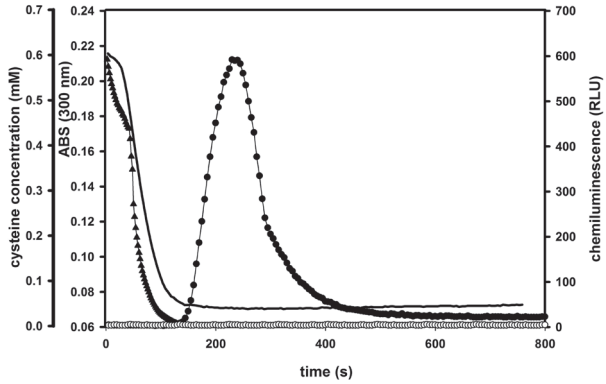

Figure 4. Generation of ROS as a result of cysteine depletion in the reaction medium.
Generation of reactive oxygen species [○] occurred when cysteine was depleted from the reaction mixture [▲]. Cysteine concentration was followed using a thiol-specific silver-mercury electrode. ROS generation was followed by intensity of chemiluminescence. Changes of Cu-His_2 absorption under the influence of cysteine were determined spectrophotometrically at 300 nm [—] . Experimental conditions were as described in Fig. 1.

Table 1. ROS generation at different concentrations of cysteine.
Amount of ROS generated was estimated as amount of chemiluminescence (area under curve) at constant Cu-His_2 concentration (final Cu^{2+} 0.1 mM).

Cysteine concentration (mM)	Area under curve (RLU) ± S.D.	RLU/ mM of cysteine ± S.D.
0.2	5453 ± 299	27266 ± 1497
0.4	12186 ± 1051	30465 ± 2628
0.5	16110 ± 641	26850 ± 1069
0.7	18113 ± 324	22641 ± 405
0.9	16979 ± 130	16979 ± 130

increased progressively with the number of aliquots (Fig. 3, Table 2). The experiment described above suggests that Cu-His_2 acts as a catalyst mediating oxidation of cysteine following each new addition of the substrate. Measured by chemiluminescence generation of ROS takes place at the end of each cycle after depletion of cysteine and is performed by active, reduced form of the copper complex and cumulated hydrogen peroxide. The proposed catalytic function of Cu-His_2 in cysteine-dependent ROS formation is in agreement with the finding that the intensity of chemiluminescence was directly proportional to the concentration of Cu-His_2 (Table 3), and that the delay of ROS synthesis was inversely proportional to the Cu^{2+} concentration.

The ROS formation always began after some delay from the time of cysteine addition. Potentiometric analysis of cysteine concentration changes in the reaction medium containing Cu-His_2 indicated that the cysteine concentration decreases during the first reaction stage and falls to zero before the ROS synthesis begins (Fig. 4). It is known that copper ions catalyze oxidation of cysteine (Kachur *et al.*, 1999; Munday *et al.*, 2004), while the highly nucleophilic thiolate anion is a potential substrate for a new complex of copper(I) with cysteine. This complex probably reduced oxygen, which resulted in the formation of $^{\cdot}O_2^-$ which accumulated in the reaction medium. The proposed mechanism is in agreement with our finding that duration of the first "silent" stage of the reaction depended positively on the cysteine concentration in the solution. As long as copper was bound to cysteine, it prevented oxygen reduction, but when the cysteine was oxidized, copper(I) was released to the medium and a rapid synthesis of ROS occurred through Fenton reaction. Copper(II) produced in this reaction was bound again into the complex with histidine.

Involvement of oxygen in ROS synthesis

Deaeration of the reaction solutions with nitrogen or helium prior to the addition of cysteine and limitation of atmospheric oxygen access by a liquid paraffin layer caused a remarkable decrease in ROS formation (Fig. 5). This observation strongly indicates that an intermediary cysteine copper complex specifically reduced oxygen. Formation of H_2O_2 in the reaction medium suggested that oxygen was reduced to the $^{\cdot}O_2^-$ radical, which eventually dismutated to H_2O_2. Our experiments showed that each addition of cysteine initiated H_2O_2 formation. We propose that oxygen is the other substrate necessary for cysteine-mediated ROS formation in the presence of Cu-His_2.

H_2O_2 synthesis in the presence of cysteine and Cu-His_2

In our oxidation model a decrease in cysteine concentration is accompanied by production of H_2O_2 as measured by an amperometric system (Fig. 4, Fig. 6). Electrometric follow up of synthesized H_2O_2 showed that the synthesis terminated at a certain level which depended on the concentration of cysteine added to the medium. Once the synthesis of H_2O_2 terminated (about 5 min after addition of cysteine), addition of a new portion of cysteine resulted in new synthesis of H_2O_2 that continued for another 2 min. In effect, the total H_2O_2 concentration in the sample increased (not shown). However, addition of cysteine before the termination of the initial synthesis of H_2O_2 halted its synthesis for a time necessary for the free cysteine to disappear from the reaction medium. Then, the synthesis of H_2O_2 started again. Each

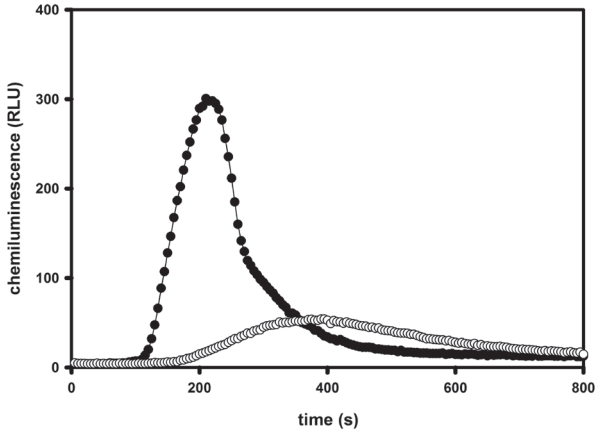

Figure 5. Influence of molecular oxygen on formation of ROS in the Cu-His_2-cysteine system.
ROS formation by cysteine and Cu-His_2 depends on oxygen access (unlimited [O] or restricted [●]) to the reaction medium. Experimental conditions were as described in Fig. 1, but oxygen was removed from the reagents (Cu-His_2 and luminol and fluoresceine) by deaeration with helium and in sample 2 the reaction medium was separated from the atmosphere by a 3-mm layer of liquid paraffin. Cysteine was injected under the paraffin layer (final concentration 0.5 mM). Chemiluminescence was measured at 5-s intervals.

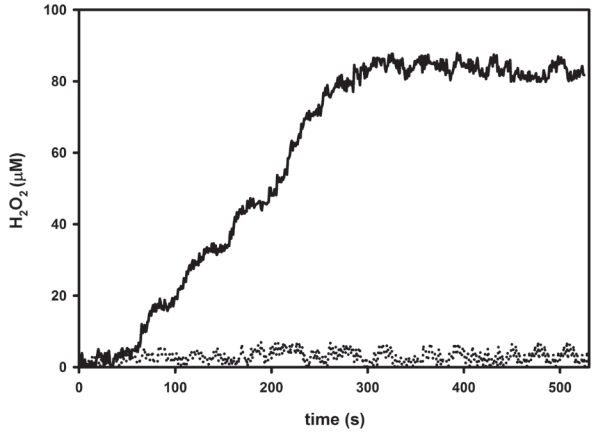

Figure 6. Generation of H_2O_2 in reaction of cysteine with Cu-His_2 at unlimited access of oxygen.
Cysteine was added to 9 ml of 55 mM phosphate buffer pH 7.4 to obtain final concentration equal to 0.5 mM. After 40 s Cu-His_2 was added to obtain Cu(II) concentration of 0.1 mM [—] . The control did not contain Cu-His_2 […] . H_2O_2 concentration was measured by amperometric method at 1-s intervals.

Table 2. Generation of ROS as a result of dosing mode of 50 µl of 6 mM cysteine (two 25-µl, five 10-µl and ten 5-µl portions) to the Cu-His_2 solution.

Cysteine portions added to Cu-His_2 solution	Height of chemi-lum-inescence peak (RLU)	Area under curve for control samples ± S.D.	Area under curve for rese-arch samples (RLU) ± S.D.	Increase of chemiluminescence (compared with control sam-ple) ± S.D.
2x 25 µl	779	9427 ± 127	9591 ± 818	164 ± 13
5x 10 µl	650	10710 ± 582	18449 ± 734	7739 ± 746
10x 5 µl	635	7583 ± 912	33664 ± 1440	26081 ± 6779

Table 3. ROS generation at different concentrations of Cu-His_2 and constant cysteine concentration (final 0.5 mM).

Cu-His_2 concentration (mM)	Area under curve (RLU) ± S.D.	RLU/ mM of Cu-His_2 ± S.D.
0.05	2802 ± 152	56040 ± 3040
0.1	5809 ± 199	58090 ± 1990
0.2	10430 ± 197	52150 ± 985

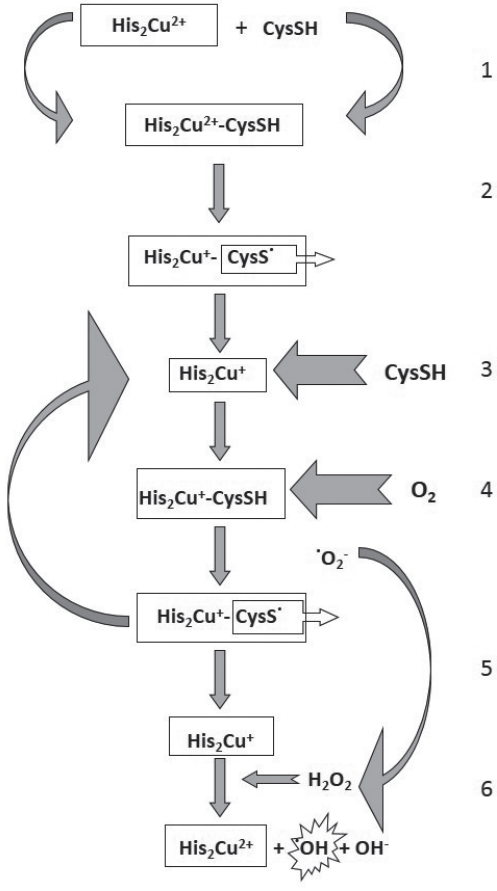

Figure 7. Proposed mechanism of cysteine-dependent ROS formation in the presence of the histidine-copper complex.
(**1**) Incorporation of cysteine to the histidine-copper(II) complex. (**2**) Internal oxidation of cysteine by copper(II). Thiyl radical of cysteine is released from the complex. It undergoes a further conversions, not shown. (**3**) Cysteine is incorporated into the histidine-copper(I) complex. (**4**) One electron oxidation of complexed cysteine by molecular oxygen. Thiyl and superoxide radicals are released. (**5**) Superoxide anion forms hydrogen peroxide as a result of spontaneous dismutation. Formation of molecular oxygen is not shown. (**6**) When cysteine is removed from the solution, hydrogen peroxide in the presence of copper(I) ions is converted to reactive hydroxyl radical in the Fenton reaction. Simultaneously, the histidine-copper(II) complex is regenerated and may react in another cycle with a new molecule of cysteine.

new addition of small amounts of cysteine caused accumulation of H_2O_2 in the reaction medium (Table 4). However, in the presence of cysteine also some reduction of H_2O_2 occurred, which was observed as a progressing decrease of the H_2O_2 electrode signal.

DISCUSSION

L-histidine binds copper, cobalt, zinc, cadmium and other bivalent transitory metal ions into stable tridentate complexes (Hofstetter *et al.*, 2011). Blood plasma practically does not contain unbound copper since it is associated with histidine residues in the binding center of ceruloplasmin or those of albumin. Cu-His_2 that constitutes about 5% of the total blood plasma copper content coordinates some of the exchangeable pool of copper in the blood (Deschamps *et al.*, 2005). However, as shown in this study, even histidine- bound copper(II) in a high affinity complex can participate in generation of free radicals in the presence of cysteine. This process includes cysteine-dependent reduction of oxygen leading to the synthesis of superoxide and hydrogen peroxide. At a physiological pH protonated thiols express low reactivity toward oxygen and hydrogen peroxide, but introduction of Cu-His_2 leads to the activation of the cysteine reducing ability, similarly as it was documented for $Cu^{2+/1+}$, $Fe^{3+/2+}$ ions in PBS solution and other transient metals (Lynch & Frei, 1997; Ullah *et al.*, 2011). One of the possible mechanisms of the cysteine- dependent ROS formation in the presence of Cu-His_2 may include reduction of copper(II) to copper(I) (Fig. 7). However, prior to this process, formation of an unstable intermediary cysteine-copper compound, either free or bound to histidine, may represent a primary product of reaction of cysteine with Cu-His_2.

The amount of generated intermediate necessary for the reduction of oxygen molecule to $^{\cdot}O_2^-$ depends on the amount of cysteine added. The superoxide radical produced in the presence of cysteine is immediately dismutated and production of H_2O_2 may be demonstrated

Table 4. H_2O_2 formation in reaction of Cu-His_2 with cysteine following sequential addition of cysteine.

Portion of cysteine added	Amount of generated H_2O_2 (µmol) ± S.D. by addition of 200 µl 6 mM cysteine to Cu-His_2
1	21.3 ± 0.9
2	35.0 ± 1.2
3	46.5 ± 1.6
4	57.0 ± 0.7
5	64.8 ± 1.0

by amperometry. When cysteine is completely depleted from the reaction medium the H_2O_2 formed can undergo Fenton reaction with complexed copper (I) to give the hydroxyl radical, which in our luminol-fluoresceine detection system generated a burst of chemiluminescence. After oxidation of copper (I) to copper(II) reconstitution of Cu-His_2 occurs and the reaction cycle may start again if a new portion of cysteine is supplied. Our results are in accord with the suggestion by Theopphanides and Anastosopulou (2002) that copper ions attached to albumin or to free amino acids in the presence of biological reductants can interact with $^{\cdot}O_2^-$ or H_2O_2 leading to the formation of hydroxyl radicals.

Oxidation of cysteine in the presence of histidine-bound copper may occur through a mechanism known from previous studies on oxidation of amino acids in the presence of transition metals forming complexes with a thiol (Kachur *et al*., 1999). Reduced cysteine (RSH) binds metal ions. The cysteine thiol group may competitively bind to histidine-copper complex to form a thiolate complex. The nucleophilic properties of the cysteine -SH residue increase when it is transformed to a thiolate anion (RS^-) (Ueland, 1995) and a thiolate-copper complex may oxidize an oxygen molecule and yield Cu^{1+}. A similar mechanism was proposed by Pecci *et al.* (1997) who demonstrated that Cu^{2+} ions form with cysteine a cuprous *bis*-cysteine complex which reduces oxygen, cytochrome and nitroblue tetrazolium. This process can also lead to reduction of copper(II) to Cu(I). The copper(I) *bis*-cysteine complex is relatively stable under anaerobic conditions, but introduction of oxygen leads to complex formation of Cu(I)–oxygen adducts which facilitate two-electron transfer to give cysteine and H_2O_2. The copper remains reduced until all cysteine is oxidized. Khossravi and Borchard (1998) studied metal-catalyzed oxidation of cysteine and histidine in the presence of H_2O_2 and proposed that combination of copper(II) and a strong reducer leads to the generation of Cu(I) which becomes a substrate for Fenton type reaction:

$$Cu^{2+} + RH^{\cdot} \text{ <----> } Cu^{1+} + R^{\cdot} + H^+$$
$$Cu^{2+} + A^{-\cdot} \text{ <----> } Cu^{1+} + A$$

The species formed in the reactions presented above can then react with O_2 to form ROS, including $^{\cdot}O_2^-$, $^{\cdot}OH$ and H_2O_2.

Another factor promoting reduction of copper(II) to copper(I) in the presence of cysteine may be the influence of histidine on the properties of the complexed copper. Histidine decreases the redox potential of the Cu(II)/Cu(I) couple, thus facilitating reduction of copper(II) and its entering into Fenton reaction (Gaubert *et* al., 2000). It means that in the presence of Cu-His_2 oxygen may undergo one-electron reduction to form $^{\cdot}O_2^-$. Therefore Cu-His_2 seems to have contrasting properties, acting both as a chelator removing Cu^{2+} from biological fluids, and as a copper(I) source that facilitates Fenton reaction and hydroxyl radical formation. Such an effect is observed in Zn,Cu superoxide dismutase (EC 1.15.1.1) which contains copper(II) bound to four imidazole residues of histidine. In ceruloplasmin, copper(II) is bound to histidine 426. However, reduction of the complexed copper may occur and in effect ceruloplasmin also has oxidative properties, which are utilized in the oxidation of Fe(II) to Fe(III) (Shukla *et al*., 2006). The influence of histidine on the susceptibility of the complexed copper to reduction increases when the histidine is located at the N-terminal position of a polypeptide chain (Ueda *et al*., 2000). Finally, free Cu-His_2 at physiological concentrations (about 25 μM) acts as a superoxide scavenger, while at higher concentrations (250 μM) it catalyzes dismutation of $^{\cdot}O_2^-$ to H_2O_2 and finally hydroxyl radical formation (Ueda *et al*., 1994).

Experiments described above indicate that cysteine is not directly involved in the synthesis of H_2O_2. On the contrary, cysteine inhibits H_2O_2 synthesis, probably by decomposing the superoxide produced by oxygen reduction. This process, however, occurs in parallel to the reaction of cysteine with Cu-His_2 yielding an intermediary product causing one-electron reduction of molecular oxygen. Such properties are shown by certain cysteine-copper(I) complexes, conceivably formed as the reaction by-products. Once cysteine is depleted from the reaction medium, $^{\cdot}O_2^-$ produced in the reaction of the intermediary product with oxygen can accumulate and dismutate to H_2O_2, which is detected in the reaction medium.

Association of copper ions with albumin, carbohydrates or enzymes can lead to in-site ROS formation *in vivo*. Site-specific metal-catalyzed oxidation that affects some specific amino acid residues located at the metal binging sites has been observed for histidine, arginine, methionine, lysine, proline and cysteine (Trigwell *et al*., 2001). Cysteine, which forms complexes with Cu(II) or Fe(III), is the most susceptible to metal-catalyzed oxidation. In effect, generation of ROS occurs within complexes of metal and cysteine, leading to oxidation of the specific local amino acid residues (Stadman, 1990). Results obtained in our studies contribute to better understanding of the mechanisms of the copper-mediated damage produced under aerobic conditions.

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