Free Rad. Res. Comms., Vol. 14, No. 3, pp. 217–225
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RESTRICTION OF THE PARTICIPATION OF COPPER IN RADICAL-GENERATING SYSTEMS BY ZINC

K. ERIN LOVERING and ROGER T. DEAN

Heart Research Institute, Missenden Road, Camperdown, Sydney, NSW 2050, Australia

(Received July 2, 1990; in revised form October 9, 1990)

Zinc was able to reduce the availability of copper for several radical-generating reactions: lucigenin-amplified chemiluminescence due to copper and hydrogen peroxide; copper-dependent ascorbate oxidation and its concomitant oxygen consumption; and copper-dependent benzoate hydroxylation. This was the case both in the presence of bovine serum albumin (when most zinc was protein-bound) and in its absence (when zinc was 'available'). Competition between zinc and copper for binding to the fluorophore calcein was also examined, and this allowed assessment of copper availability in several circumstances. Competition between copper and zinc for binding to biological components seems to be a rather general phenomenon, and thus zinc is commonly a protective entity, restricting free radical generation.

KEY WORDS: Copper; zinc, free radicals, protection.

INTRODUCTION

It has long been proposed that amongst the functions of zinc are membrane stabilisation,[1] and protection against free radical damage.[2,3] Zinc is protective against some free radical stresses in cellular systems,[4] and there are epidemiological indications of a similar role in restricting the development of atherosclerosis and cancer.[5] Very recently a clear experimental demonstration of the protective role of zinc has been provided:[6] zinc could protect *E. coli* against copper-mediated paraquat-induced damage. This paraquat-induced damage involves radical generation and the authors propose that copper, bound to a variety of biological molecules, can localise radical-generating reactions upon them. Zinc is envisaged to displace copper from such sites, or to form ternary complexes with copper and the binding molecule, such that free radical generation is restricted.

The idea that zinc competes with copper for binding to critical biological molecules has been expressed in the literature for a long time (reviewed [7]) but has regained force recently because of an increased emphasis on the importance of the site-specific damaging mechanisms of transition metals.[7,8] Nevertheless, rather few well defined instances of competition between zinc and copper for biological binding sites influencing radical generation have been presented. Amongst those in the literature are demonstrations of zinc inhibiting lipid peroxidation *in vitro*,[2] competing with copper for participation in thiol autoxidation systems,[9] and forming ternary complexes of copper, zinc and carnosine.[10] Zinc and copper can also compete for binding to

Correspondence to Roger T. Dean at the above address.

metallothionein (MT), and though MT is thought to have a primary function in protecting against heavy metal toxicity, MT may also have a role in transport of Cu and Zn.[11] Similarly caeruloplasmin can bind both metals, and may be particularly involved in transport and sequestration of copper.[12]

Zinc concentrations in human plasma are around 15 μM,[13,14] but they may reach much higher levels, such as 200 μM in human erythrocytes.[14] In plasma, and perhaps inside cells, most zinc is bound to proteins (in plasma, notably α-2-macroglobulin and albumin:[14]). The following experiments have therefore been undertaken with zinc concentrations from 5 μM up to 1 mM, from below to above the maximum likely *in vivo* concentrations. They have also been undertaken in the presence of protein, such that the added metal in the system would be protein bound in most cases.

We have studied four assay systems with defined radical-generating mechanisms in which copper is an essential participant. We have found that both in the presence and absence of protein, zinc restricted the participation of copper in these systems, and hence overall radical generation. We show that this can result from competition between zinc and copper for binding sites on both small and large biological molecules, and suggest that this is a general phenomenon.

MATERIALS AND METHODS

All materials were of highest purity commercially available, and were obtained from Sigma. In particular, bovine serum albumin (BSA) was Sigma Fraction V (#A6793, Sigma Chemical Co., USA).

Since the experiments involved the addition of relatively large quantities of copper to various systems and hence standardised the available copper, it was necessary to check whether iron concentrations might be sufficient to affect the reactions followed. Iron was assayed by formation of coloured complexes with ferrozine, after reduction. It was found that our 10 mM-phosphate buffer contained less than 0.1 μM iron, while our 10 μM-BSA contributed less than 0.5 μM iron. Other reagents contained lower quantities of iron, near the limits of sensitivity of the assay, and thus the highest iron concentration in any of our incubations was less than 0.7 μM, allowing 0.1 μM for all the other reagents.

Method of Addition of Zinc

The buffering system throughout was 10 mM-sodium phosphate, pH 7.2. Since zinc was added to the standard radical generating systems outlined below as zinc sulphate, which increases the solubility of the available copper, it was necessary to standardise the sulphate concentration. This was done by including 5 mM-sodium sulphate in each system except for oxygen consumption (where a sulphate concentration of 10 mM was used). Thus sulphate concentrations were at the most increased by 20 % above these levels.

Lucigenin amplified chemiluminescence

This was conducted essentially as described previously, using copper and hydrogen peroxide in a phosphate buffered system.[15] The possibility that zinc reduces chemiluminescence yield by interfering with lucigenin rather than radical generation was

excluded by two approaches. Firstly, in the concentrations studied, zinc had no effect on the fluorescence spectrum of lucigenin. Secondly, when chemiluminescence was generated from lucigenin by a metal-independent system (using the thermolabile radical-generator, 2,2′-azobis [2-amidino-propane] dihydrochloride [AAPH]), zinc had insignificant effects on luminescence yield.

Ascorbate Oxidation

This was conducted with the following standard concentrations, essentially as described.[16] Ascorbate was added at 0.1 mM. Oxygen consumption was measured by a Yellow Springs oxygen electrode, with an initial ascorbate concentration of 0.17 mM.[17]

Benzoate hydroxylation

was conducted as described previously.[15] The final concentrations were hydrogen peroxide, 5 mM; copper sulphate, 50 μM; sodium benzoate, 1 mM; sodium sulphate, 5 mM; sodium phosphate buffer pH 7.2, 10 mM. The reaction was conducted at 25° C, in a final volume of 4 ml. The conversion of benzoate into fluorescent salicylates was measured (excitation 308 nm, emission 410 nm).

Metal Detection by Calcein Fluorescence

The fluorophore calcein was used to monitor available copper.[18] The standard assay utilised 2 μM-calcein, whose fluorescence is quenched by copper in a dose dependent manner (virtually complete quenching with 4 μM copper). Readings were routinely taken 5 minutes after assembly of the mixtures. Copper bound to protein, or to EDTA, was unavailable for binding to calcein within incubations of up to 1 hour. Zinc could also bind to calcein but produced relatively little quenching of fluorescence in the same concentration range (1 μM to 20 μM).

Presentation of Results

All data presented are from single experiments, representative of several.

RESULTS AND DISCUSSION

Zinc restricts the participation of copper in radical-generating systems without protein

Figure 1 shows that zinc produces a dose dependent inhibition of chemiluminescence generated by copper and hydrogen peroxide. With 50 μM-copper, there was detectable inhibition by 50 μM-Zn and virtually complete inhibition with 500 μM-Zn and higher concentrations. Thus Zn could compete with low molecular weight Cu for crucial component(s) of this reaction. The chemiluminescence detectable in Figure 1 (and also Figure 3) prior to addition of copper, is probably due to small quantities of copper present in the reagents, and is also inhibited by zinc in a dose dependent manner.

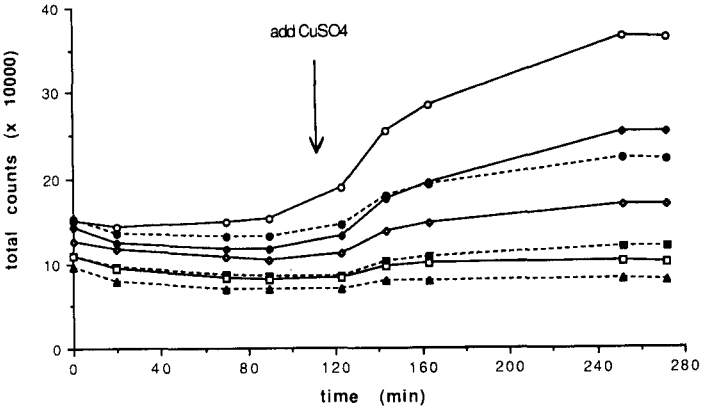

FIGURE 1 Inhibition by zinc of the reaction of copper and hydrogen peroxide, as assessed by chemiluminescence. Chemiluminescence amplified by lucigenin (154 μM) was determined (0.1 minute count) in a system comprising: 10 mM-sodium phosphate buffer (pH 7.2), 50 μM-$CuSO_4$, 5 mM-Na_2SO_4, and hydrogen peroxide, 5 mM. Zinc sulphate was added at the following concentrations: —○— 0; —◆— 50 μM; --●-- 100 μM; —◇— 200 μM; --■-- 500 μM; —□— 800 μM; --▲-- 1 mM. Reaction volume was 1 ml, and the chemiluminescence was measured at room temperature in an LKB scintillation counter used in the out-of-coincidence mode. Copper was added (arrow) after the fourth chemiluminescent count had been taken. The rise in chemiluminescence started thereafter although the graphs (in this figure and Figure 3) are drawn as if the change in rate commenced at the point prior to copper addition. Each data point is the average of duplicates.

Figure 2 illustrates a comparable experiment in the copper-dependent ascorbate oxidation system. The results were similar in that, with copper at 50 μM, there was detectable inhibition of oxidation by 50 μM-Zn, and inhibition was progressively increased as the zinc concentration was raised to 1 mM. Nevertheless, even at 1 mM-Zn (i.e. at Zn:Cu 20:1) there was still ascorbate oxidation, which could be completely inhibited by the addition of EDTA. Thus the ascorbate oxidation observed was still metal-catalysed. Presumably therefore, zinc competes with copper for binding to ascorbate less efficiently than it competes for the critical component of the chemiluminescence system.

Zinc also restricts the participation of copper in radical-generating systems in the presence of protein

To mimic more closely the *in vivo* conditions, in which at least extracellularly, most zinc is protein bound, we have also studied the influence of zinc on the two systems described already but with protein present. Figure 3 shows that the addition of BSA (10 μM) to the copper-hydrogen peroxide chemiluminescence system enhanced radical generation. We have studied this phenomenon in more detail elsewhere[19] and, like the enhancement by Hepes,[20] it is due to formation of copper complexes with the added component (in this case with protein: for review of recent studies on copper binding to albumins, see[21]). The complexes increase the solubility and hence availability of copper, and perhaps also change its redox potential, so as to permit it to participate more extensively. Thus the maximum chemiluminescence in the positive control condition in Figure 3 is approximately twice that of Figure 1, and is reached more rapidly. However, the addition of zinc again produced a dose-dependent inhibi-

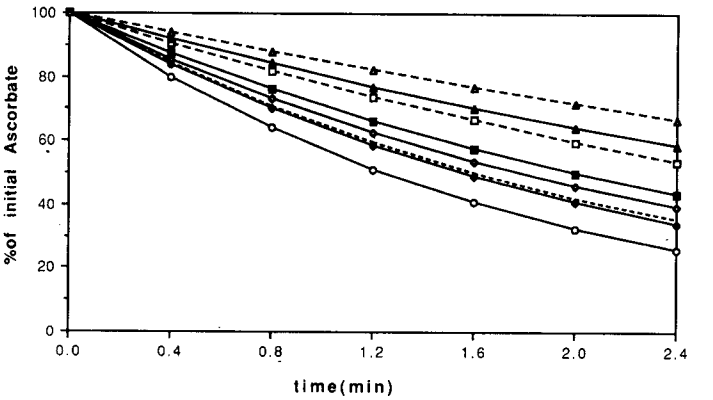

FIGURE 2 Inhibition of copper-dependent ascorbate consumption by the addition of zinc in the absence of protein. Ascorbate consumption was followed as decline in absorbance at 265 nm at room temperature. The system comprised: 100 μM ascorbate, 10 mM-sodium phosphate buffer (pH 7.2), 50 μM-$CuSO_4$, 5 mM-Na_2SO_4. Zinc sulphate was added at the following concentrations: —○— 0; —◆— 5 μM; ------ 10 μM; —◇— 25 μM; --■-- 50 μM; —□— 200 μM; --▲-- 500 μM; --△-- 1 mM. Each line indicates successive readings on a single incubation.

tion of chemiluminescence. In this case, even with 1 mM-Zn the chemiluminescence was only reduced by about 50 %. The kinetics were unchanged (in that the peak of the reaction was still reached very rapidly); suggesting that Zn was inhibiting reactions involving some of the protein-bound Cu, as well as inhibiting the reactions of low MW Cu (as shown earlier: Figure 1).

When BSA (10 μM) is added to the copper-dependent ascorbate oxidation system the rate of oxidation is virtually unchanged (Figure 4 vs Figure 2; no Zn control conditions in each case). Copper is known to form a complex with ascorbate, and so available low MW Cu is probably sufficient for maximal ascorbate oxidation in the absence or presence of protein. Much copper is bound to available protein (as judged

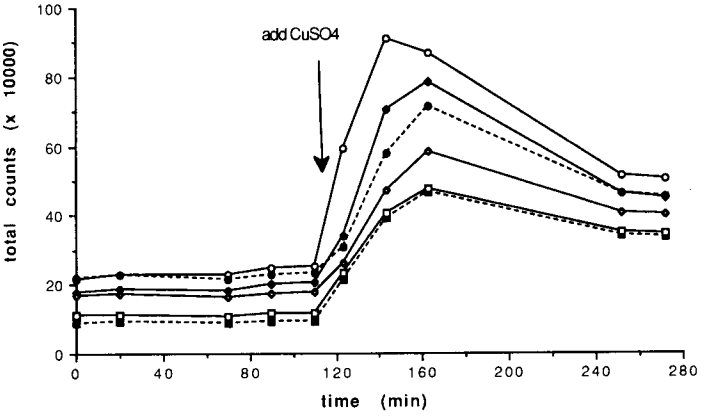

FIGURE 3 Inhibition by zinc of the reaction of copper and hydrogen peroxide in the presence of protein. The experiment was performed as for Figure 1, with bovine serum albumin at 10 μM (0.68 mg/ml), and with zinc at the following concentrations: —○— 0; —◆— 100 μM; --●-- 200 μM; —◇— 500 μM; --■-- 800 μM; —□— 1 mM. Data are average of duplicates.

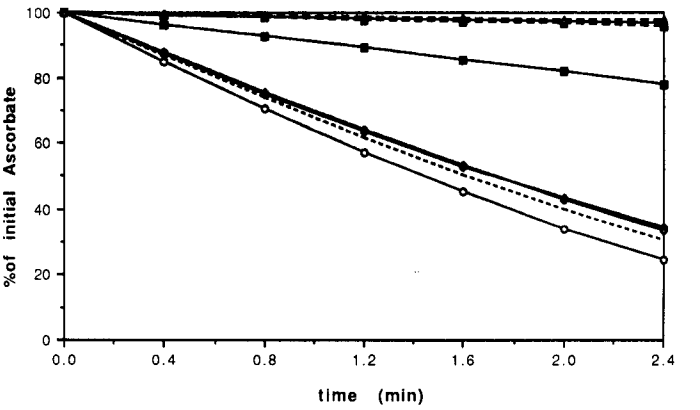

FIGURE 4 Inhibition of copper-dependent ascorbate consumption by the addition of zinc, in the presence of protein. The experiment was performed as in Figure 2, with bovine serum albumin at 10 mM. Zinc was added at the following concentrations: —○— 0; —◆— 5 μM; ------ 10 μM; —◇— 25 μM; --■-- 50 μM; —□— 200 μM; --▲-- 500 μM; --△-- 1 mM. Each line indicate successive readings on a single incubation.

by the calcein assay) but presumably low MW Cu remains in excess for the reaction. When zinc is present, it again restricts the availability of copper for the oxidation. In this case, inhibition is complete at the highest zinc concentrations studied (200 μM and above). Zinc is presumably primarily competing with low MW Cu for the reaction with ascorbate. This competition is presumably more effective than in the absence of protein because the free copper concentration is lowered by the presence of protein.

Other demonstrations of competition between Zn and Cu and its relevance

In Figure 5 the oxygen consumption during ascorbate oxidation in the presence and absence of protein is shown. These results complement and confirm those of Figures

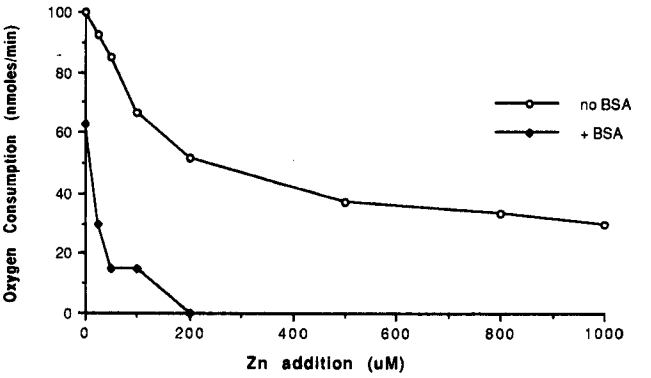

FIGURE 5 Inhibition by zinc of oxygen consumption during copper-dependent ascorbate oxidation. The reaction comprised 167 μM ascorbate, 10 mM-sodium phosphate buffer (pH 7.2), 50 μM-$CuSO_4$, 10 mN-Na_2SO_4. Oxygen consumption was followed either in the presence of bovine serum albumin (10 μM) or in its absence. Zinc was used at concentrations up to 1 mM.

2 and 4, and show that zinc can completely prevent oxygen consumption in the presence of BSA but only partially inhibit in its absence. In the absence of zinc, the oxygen consumption is lower when BSA is present than when it is absent, presumably because of the binding of some copper to the protein which consequently reduced the activity.

Benzoate hydroxylation by copper and hydrogen peroxide was also studied (data not shown in detail). With copper at 50 μM, zinc could inhibit in a dose dependent manner, and there was approximately 45 % inhibition with 500 μM-Zn. In the presence of 10 μM-BSA, zinc inhibited more extensively, by up to 75 % with 350 and 500 μM-Zn. This system thus behaves similarly to the ascorbate oxidation system, presumably again because only low concentrations of free copper are needed for maximal activity, and also because copper and zinc complex with benzoate.

We propose that all of these inhibitory actions of zinc upon copper-dependent oxidations involve competition for binding to critical components of the systems. To illustrate this further we examined competition between Zn and Cu for binding to the fluorophore calcein (data not shown). With 2 μM-calcein, zinc slightly quenched fluorescence, such that quenching was maximal (approx. 38 %) at 5 μM zinc and above. In contrast, 4 μM-Cu abolished virtually all the fluorescence of 2 μM calcein.

With 2 μM copper present, calcein fluorescence was quenched by approximately 93 %. When such complexes were subsequently exposed to 20 μM-Zn (Cu:Zn 1:10), quenching was essentially unchanged after 5 min incubation although there was a subsequent slow recovery of fluorescence which was not studied in detail. When complexes of Zn (20 μM) and calcein (2 μM) were preformed and then exposed to 2 μM-Cu, the quenching was only 65 % after 5 minutes. Thus confirming that Zn and Cu compete, although relatively slowly, for calcein. Also it was shown that protein could sequester copper so it was unavailable for calcein; and that the addition of zinc could release small quantities of bound copper, with a subsequent increase in calcein quenching.

Thus zinc and copper compete for binding to many different biological molecules, small and large. We have additionally found that copper will bind to preformed liposomes containing phosphatidylserine, and is thereby made less able to oxidise ascorbate, or to bind to calcein. Again zinc competes for these copper binding sites, increasing the availability of copper for binding to calcein (data not shown).

We therefore propose that the competition between zinc and copper is a general process, and hence support the view that zinc has an important protective role in free radical biochemistry, by limiting the availability of transition metals for redox reactions. In agreement with this view, zinc is present in most biological fluids at somewhat higher concentrations than copper. For instance, in rabbit plasma copper is around 9 μM and zinc around 13, and the concentrations are susceptible to dietary manipulation.[22] In rat liver and lung, zinc to copper molar ratios are approx 5:1 and 13:1, respectively.[23] Zinc is also concerned with control of metallothionein which has other protective actions which are outside the scope of this paper([23,24] reviewed[11]) and there has recently been much interest in "zinc-fingers" in certain regulatory proteins.

Survivors of myocardial infarction have elevated Zn:Cu ratios in head hair, consistent with the view that zinc is protective against copper mediated oxidative actions[5] and certain pathologies may be associated with a decrease in the Zn:Cu ratio.[25] In such pathological extracellular fluids, for example some inflammatory exudates,[26] and possibly in the atherosclerotic plaque, the radical-generating actions of copper may be unfortunately enhanced.

Acknowledgements

We thank Cathy Kneale for able research assistance. The Heart Research Institute acknowledges support from the Government of NSW and the Commonwealth Government of Australia, the National Heart Foundation of Australia and many Corporate donors. K.E. Lovering is supported by BHP Community Trust.

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Accepted by Prof. J.M.C. Gutteridge