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THE SOD LIKE ACTIVITY OF COPPER:CARNOSINE, COPPER:ANSERINE AND COPPER:HOMOCARNOSINE COMPLEXES

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Carnosine, anserine and homocarnosine are natural compounds which are present in high concentrations (2–20 mM) in skeletal muscles and brain of many vertebrates. We have demonstrated in a previous work that these compounds can act as antioxidants, a result of their ability to scavenge peroxyl radicals, singlet oxygen and hydroxyl radicals. Carnosine and its analogues have been shown to be efficient chelating agents for copper and other transition metals. Since human skeletal muscle contains one-third of the total copper in the body (20–47 mmol/kg) and the concentration of carnosine in this tissue is relatively high, the complex of carnosine:copper may be of biological importance. We have studied the ability of the copper:carnosine (and other carnosine derivatives) complexes to act as superoxide dismutase. The results indicate that the complex of copper:carnosine can dismute superoxide radicals released by neutrophils treated with PMA in an analogous mechanism to other amino acids and copper complexes. Copper:anserine failed to dismute superoxide radicals and copper:homocarnosine complex was efficient when the cells were treated with PMA or with histone-opsonized streptococci and cytochalasine B. The possible role of these compounds to act as physiological antioxidants that possess superoxide dismutase activity is discussed.

KEY WORDS: Superoxide dismutase, copper, carnosine, anserine, homocarnosine.

INTRODUCTION

Carnosine (β-alanyl-L-histidine), anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) are natural compounds which are synthesized in animals and humans and are present in high concentrations in brain and muscles (2–20 mM).[1-3] Several suggestions have been made concerning the role of these compounds, but none have satisfactorily explained their biological functions. Carnosine has been postulated to act as a buffer to neutralize lactic acid produced in skeletal muscles that is undergoing anaerobic glycolysis.[4] Other suggestions include the possible role of these compounds as putative neurotransmittors in the olfactory bulbs and carnosine has been suggested as a physiological activator for myosin ATPase, or in the regulation of other enzymes.[5,6] Hartman and Dahal have shown that carnosine is an efficient singlet oxygen scavenger, quenching singlet oxygen more effectively than does an equimolar concentration of histidine.[7] Bondarenko *et al.*[8] have shown that homocarnosine protects against the convulsions obtained by the exposure of animals to hyperbaric oxygen and that its content decreases in rabbits after they have been exposed to hyperoxia. Carnosine protects rabbit hearts after ischemia. We have suggested in a previous work that these compounds may act as natural antioxidants in brain and muscle.[9]

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Carnosine and anserine have also been shown to be very efficient copper chelating agents *in vivo*, and it has been suggested that they may play a role in Wilson's disease. It has also been suggested that they may play a role in copper metabolism *in vivo*.[3]

Based on the high concentrations of these compounds and their ability to bind transition metals such as copper, we suggest in this research that these complexes may posses SOD activity in an analogous mechanism to other amino acid and copper ions.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma, blood was obtained from Hadasaah medical center.

Blood Neutrophils (PMN's)

Human blood was obtained from healthy donors. PMN's were isolated on a Ficoll Hypaque gradient, followed by dextran sedimention as described elsewhere.[10] Such preparations contain more than 95% viable PMN's. Erythrocytes were removed by treatment with hypotonic saline. The PMN's were resuspended in Hank's balanced salt solution (HBSS) buffered with 1 mM HEPES buffer, pH 7,4.[10] In some cases the PMN's were suspended in HBSS-HEPES buffers containing 10 mM sodium azide. All the samples contained small concentrations of albumin and other small molecules present in the blood.

Stimulation of the neutrophils

PMN's ($1-5 \times 10^6$/ml) in HBSS were treated with (A) phorbol miresitate acetate (PMA) 1 mM, or with (B) streptococci A (200 KU/ml) opsonized with histone type II A (1%). In some experiments cytochalasine B (cy. B) (2.5 μg/ml) was added to the reaction mixture. The complexes to be tested were added followed or previous to the addition of the various compounds described above.

Measurement of the superoxide radicals (O_2^-)

Superoxide was determined in the stimulated PMN's by following the reduction of cytochrome C (80 μM type III, Sigma) according to the method described by Babior.[11] The reaction mixture contained PMN's (15×10^6/ml), ligands (as described before), cytochrome C and additives to a final volume of 1 ml. The reaction mixtures were incubated in a water bath at 30°C. At various time intervals samples were taken, centrifuged at 1000 g for 5 min. The optical density of the supernatant fluids was read at 550 nm. The amount of the superoxide radicals was calculated from the extinction coefficient ($\varepsilon_{550} = 2.1 \times 10^4$) and was expressed as nmoles per number of cells per 10 min. The O_2^- observed in the control experiments was found to be 38–48 nmoles/10 min (see results).

Measurements of luminol-dependent chemiluminescence (LDCL)

LDCL was induced in the PMN's by PMA or by streptococci opsonized with histone.

Luminol 5 μl (2 mg/ml) was added to the reaction mixture. The solutions were left for incubation for 30 sec. and then the various tested copper complexes were added. The tubes were vortexed and immediately placed in a Lumak luminator.

Determination of hydrogen peroxide

Hydrogen peroxide was determined according to the method of Thurman *et al.*[12]

Preparation of the copper complexes

Copper:carnosine, copper:anserine and copper:homocarnosine were prepared by mixing the carnosine, anserine or homocarnosine (3 mM) and copper (6.2 μM), which was found to be non-toxic to the PMN's (see results), prior to the addition to the reaction mixtures. The toxicity of the various complexes to the PMN's was tested by following the release of the enzyme lactate dehydrogenase.

All the experiments were carried out at least 3 times and the results show the mean of the various repetitions.

RESULTS

The first experiments dealt with the concentration range of copper ions which is not toxic to the cells. Various copper concentrations were introduced to the reaction mixture containing PMN's and PMA as inducer. The superoxide radicals production from the induced PMN's was not effected up to a concentration of 6.5 μM copper sulphate (results are not shown). When 20 μM of copper sulphate was used, 12% decrease in the superoxide radicals production was detected. In all the experiment we have used two different methods for the stimulation of the PMN's. The first method

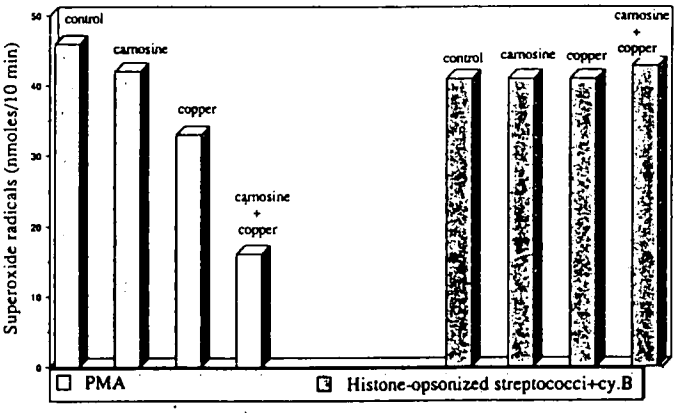

FIGURE 1 The effect of copper:carnosine complex on the superoxide radicals released by PMN's treated with PMA or with histone-opsonized streptococci. The reaction was initiated by addition of PMA (1 mM) or streptococci (200 KU/ml) opsonized with histone (type IIA (%)) and cytochalasine B. to PMN's (4–5 $\times$ 10^6 cells/ml). Freshly prepared copper:carnosine complex (6.2 μM:3 mM) was added to the reactuion mixture as described. The generation of superoxide radicals was monitored by following the reduction of cytochrome *C* as described in the methods after 10 min of incubation.

included the use of a soluble inducer, the PMA, and the second method included the use of insoluble particles, the histone opsonized streptococci and cytochalasine B.

Figure 1 shows the superoxide radicals released from the induced PMN's and various treatment with carnosine, copper and their combinations. The PMN's which were triggered with the insoluble particles have shown no effect of either carosine alone, copper alone or the complex of copper:carnosine. However, the superoxide radicals released from the PMN's which were exposed to the soluble inducer were significantly effected from the addition of the complex of copper:carnosine. For example, while carnosine alone decreased the superoxide production by 9% and copper alone by 29%, the combiantion of copper and carnosine resulted in a decrease of 65% in the superoxide radicals detected (Figure 1).

Anserine was tested in the salt form as anserine nitrate. The results as shown in Figure 2 indicate that there was no decrease in the superoxide radicals production when the complex copper:anserine was present, when PMA was used as inducer or when the PMN's were induced with the histone opsonized streptococci and cy. B. For example, when the PMN's were treated with PMA, aanserine decreased the superoxide production by 10%, copper alone by 7.5% and the combination of copper and anserine by 17.5%. The complex copper:anserine showed 45% decrease in the superoxide production when the PMN's were treated with histone-opsonized streptococci similar to the effect of copper ions alone (43% of inhibition).

Significant effect on the superoxide radicals released from the PMN's was observed when the cells were treated with the complex copper:homocarnosine. Figure 3 shows a reduction of 73% in the superoxide radicals detected whent he cells were induced with PMA and in the presence of copper:homocarnosine. When the cells were exposed to homocarnosine alone, no effect was recorded. A decrease of 16% was observed when copper ions alone were present in the reaction mixture. Similar results were obtained when the cells were induced with histone-opsonized streptococci and Cy. B. A decrease of 56% in the superoxide radicals was detected when the complex copper:homocarnosine was introduced to the cells treated with the histone-opsonized

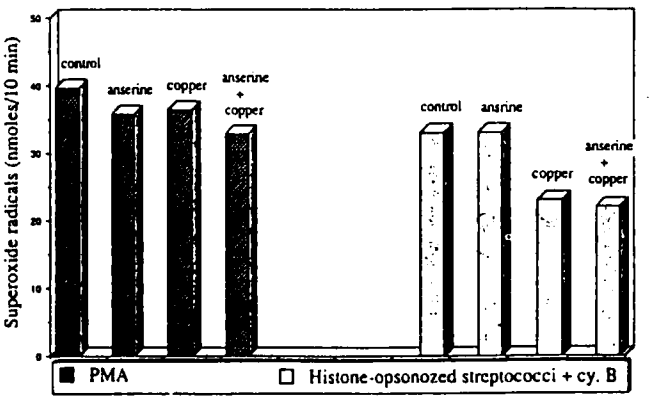

FIGURE 2 The effect of copper:anserine complex on the superoxide radicals released by PMN's treated wth PMA or with histone-opsonized streptococci. The reaction was initiated by addition of pMA (1 mM) or streptococci (200 KU/ml) opsonized with histone (type IIA (%)) and cytochalasine B. to PMN's (4–5 × 10^6 cells/ml). Freshly prepared copper:anserine complex (6.2 μM:3 mM) was added to the reaction mixture as described. The generation of superoxide radicals was monitored by following the reduction of cytochrome *C* as described in the methods after 10 min of incubation.

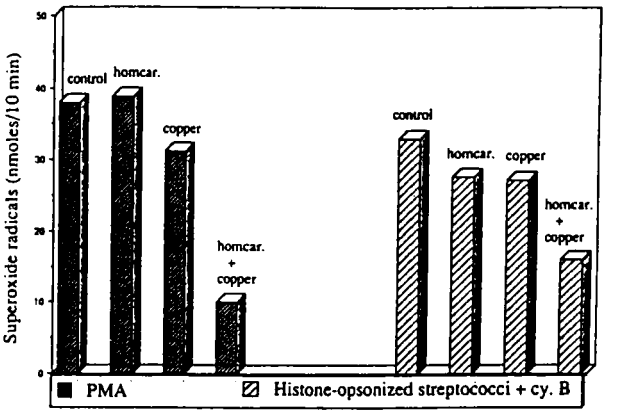

FIGURE 3 The effect of copper:homocarnosine complex on the superoxide radicals released by PMN's treated with PMA or with histone-opsonized streptococci. The reaction was initiated by addition of PMA (1 mM) or streptococci (200 KU/ml) opsonized with histone (type IIA (%)) and cytochalasine B. to PMN's ($4–5 \times 10^6$ cells/ml). Freshly prepared copper:homocarnosine complex (6.2 μM:3 mM) was added to the reaction mixture as described. The generation of superoxide radicals was monitored by following the reduction of cytochrome *C* as described in the methods after 10 min of incubation.

streptococci, while only 20% reduction in superoxide radicals was detected when copper alone or homocarnosine alone were added to the reaction mixture.

Homocarnosine alone decreased the chemiluminescence observed by 12% and carnosine alone by 18%. The complex of copper:homocarnosine decreased the chemiluminescence observed by 55%. Similar results were obtained when the complex of copper:carnosine was used. No decrease in the chemiluminescence was recorded when anserine or the complex of copper:anserine was used (data not shown).

We have also measured the production of hydrogen peroxide in the various systems. No decrease in the production of H_2O_2 was recorded and in some cases a slight increase in hydrogen peroxide production was detected (data not shown).

DISCUSSION

The broad definition of biological antioxidant includes compounds which can prevent oxidative damage to lipids, proteins, DNA, and other essential macromolecules. Most antioxidants are specific and can provide only one type of protection (i.e. blocking free radical initiation, removing oxidants from biological targets, reacting with the reactive species thus sparing the biological target, transforming a reactive species to a non-reactive species, stabilizing membranes, and acting directly by removal of mediators which catalyse free radical damage[9]). The defence mechanisms against oxidative stress can be divided into several categories in which the enzymatic category is of major importance. The enzymes superoxide dismutase, catalase and peroxidase serve in the first line of defence against reactive oxygen species. However, since the radicals themselves are relatively small molecules they can easily escape the enzymes and can cause biologtical damage in targets where the defence enzymes have no access. Other small antioxidants, such as ascorbic acid, vitamin E, glutathione etc., can protect biological target against oxygen species. The superoxide radical is con-

sidered to be a toxic specie which can damage proteins, fatty acid and other essential molecules. Beside the enzyme superoxide dismutase, there are other small molecules which posses SOD-like activity *in vitro*, usually small complexes of amino acid and copper ions. In this research we have studied the possibility that natural histidine-related compounds such as carnosine, anserine and homocarnosine might serve as SOD-like molecules when complexed with copper ions.

One of the mecahnisms by which antioxidants can protect their biological targets from oxidative stress is the chelation of transition metals such as copper and iron thus preventing them from participating with peroxides in the deleterious Fenton reaction. Carnosine, homocarnosine and anserine were shown to be efficient copper chelating agents which can bind copper ions *in vivo*.[3] Human skeletal muscle contains one third of the total copper in the body (20–47 μmol kg^{-1}), thus the presence of these compounds in this tissue, at high concentrations might indicate their physiological role as a chelating agents of copper ions. Copper concentrations in the olfactory bulbs were found to be 50 μM, while carnosine, homocarnosine and anserine concentrations are in the millimolar range, suggesting the possibility for the chelation of copper by these molecules. We have chosen to perform our experiment in a relatively biological system, in which, the superoxide radicals were produced by polymorphonuclear cells treated with soluble or with nonsoluble inducers. The results indicate that these complexes possess SOD acitivity as shown in Figure 1, where copper:carnosine was used and in Figure 3 where copper:homocarnosine was used. The complex of copper:anserine has not shown any significant activity in decreasing the release of superoxide radicals from the PMN's triggered with either PMA or with histone-opsonized streptococci and cy. B. (Figure 2). The anserine molecule contain a methyl group on the imidazole ring (β-alanyl-3-methyl-L-histidine)[9] which might interfere with the binding of the copper and so prevent its participation in the SOD enzymatic activity. Similar results were obtained from the experiments with LDCL. The complex of copper:carnosine was efficient in reducing the superoxide radicals, only when the cells were stimulated with PMA. This might indicate that the complexes can prevent the superoxide production by interfering with the induction mechanisms of the superoxide radicals and that they do not serve as catalysts in the dismutation of the superoxide radicals themselves. However, the increase in the production of H_2O_2 may suggest an analogues mechanism to that of the enzyme SOD. Another possibility that has to be resolved, is that the complexes may serve as scavengers of the superoxide radicals rather than as catalysts. The complex copper:homocarnosine has demonstrated a strong SOD-like activity even when the cells were treated with histone-opsonizes streptococci and cy. B. which may indicate its biological significant as a natural SOD-like molecule in the brain, a tissue with high oxidative stress, and with high concentration of homocarnosine and copper ions. Further studies are needed in order to confirm the mechanism of action of the various complexes and to determine the rate constants for the dismutation reaction of the complexes.

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