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HYPOCHLOROUS ACID-INDUCED ZINC RELEASE FROM THIOLATE BONDS: A POTENTIAL PROTECTIVE MECHANISM TOWARDS BIOMOLECULES OXIDANT DAMAGE DURING INFLAMMATION

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It has been proposed that metalloprotein zinc mobilization mediated by hypochlorous acid (HOCl) may induce cell injury (see H. Fliss and M. Ménard (1991), Archives of Biochemistry and Biophysics, **287**, 175-179). In the present paper, we have demonstrated using a dimercaptopropanol-zinc complex that, once released from thiolate bonds by HOCl, zinc can exert a significant antioxidant effect on both linolenic acid and deoxyribose oxidation induced by iron. In these experimental conditions, however, the antagonism towards deoxyribose oxidation is notably less than that towards linolenic acid peroxidation, thus suggesting a more specific inhibitory effect of zinc on iron-mediated oxidant damage when polyunsaturated fatty acids represent the oxidizable substrate. The antioxidant effects of zinc are strictly related to the "free" form; indeed, the dimercaptopropanol-zinc complex per se is stimulatory even on biomolecules oxidant damage, apparently as a result of the prooxidant interaction of the thiol compound with iron. In light of these results, it may be proposed that the zinc released from thiolate bonds by HOCl could specifically limit tissue oxidative burden in pathological conditions involving neutrophil accumulation and activation, such as inflammation and ischemia-reperfusion.

KEY WORDS: Zinc; hypochlorous acid; antioxidant; iron; lipid peroxidation.

INTRODUCTION

Hypochlorous acid (HOCl) is probably the most toxic of the oxidants produced by activated neutrophils, inducing oxidative alterations of proteins, thiols, nucleotides and polyenoic acids.[1] Moreover, HOCl may favour the mobilization of powerful oxidizing transition metals, such as iron and copper, via denaturation of iron and copper-containing proteins.[2,3] In such a context, a HOCl-mediated zinc release from metalloproteins has also recently been reported,[4] though the pathophysiological significance of this phenomenon appears not yet fully known. Fliss and Ménard[4] have speculated that the zinc released from thiolate bonds by HOCl may amplify biomolecule damage during inflammation. However, in light of the zinc antioxidant

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properties,[5] a protective biological role for the metal delocalized from binding sites by prooxidant forces could be hypothesized.

In the present paper, therefore, we have specifically investigated the effect of zinc released from thiolate bonds by HOCl on the oxidative damage of polyunsaturated fatty acids and deoxyribose. The results show that, once mobilized from thiolate bonds, zinc can exert a significant antioxidant action, thus suggesting potential beneficial effects of the metal on neutrophil-mediated oxidant injury during inflammation.

MATERIALS AND METHODS

Reagents

4-(2-pyridylazo)resorcinol (PAR), 2,3 dimercaptopropanol (DMP), sodium hypochlorite, 2-deoxyribose, linolenic acid and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co., St Louis, MO. All other reagents were of the highest grade commercially available.

The DMP-zinc complex was prepared under conditions of a 2:1 molar excess of DMP over Zn^{2+}, by incubating DMP and $ZnCl_2$ in distilled water for 30 min at room temperature;[4] the complex used for experimental purposes was formed by 22 μM DMP and 11 μM Zn^{2+}.

HOCl-induced Zinc Mobilization from the DMP-zinc Complex

Reaction mixtures, in 1.0 ml cuvettes, contained 100 μM PAR and 22 μM DMP complexed with 11 μM Zn^{2+}, in 40 mM Hepes buffer, pH 7.0.[4] It should be noted that the 2:1 PAR-Zn^{2+} complex formation is not influenced by the use of phosphate buffers, as reported also by Hunt *et al.*[6] When required, HOCl was added at physiologically relevant final concentrations of 50, 100 and 200 μM. HOCl was freshly prepared, and its concentration was determined spectrophotometrically using a molar extinction coefficient of 350 M^{-1} at 290 nm.[4] Absorbance values at 500 nm, due to PAR-zinc complex formation, were recorded in basal conditions and after 5 min incubation at 37°C with stated HOCl concentrations on a double beam Varian DMS 200 spectrophotometer. Zinc concentrations were calculated using a molar extinction coefficient of 6.5×10^4 M^{-1} cm^{-1}[4].

Iron-driven Linolenic Acid and Deoxyribose Oxidation

For linolenic acid oxidation mediated by iron, a phosphate buffer (i.e., 0.18 M phosphate buffered saline, pH 7.4) was used to favour Fe^{2+} autooxidation and oxidizing radical generation. To the DMP (22 μM)-zinc (11 μM) complex, preincubated for 5 min at 37°C with 200 μM HOCl in the phosphate buffer, a suitable amount of ethanol-solubilized linolenic acid was added, thus obtaining 4 mM and 10% final concentrations of emulsified fatty acid and ethanol, respectively. In these experimental conditions and in light of the results obtained with the DMP-zinc complex in the presence of 200 μM HOCl and 100 μM PAR, the concentration of "free" zinc was about 11 μM. Lipid peroxidation was induced by 11 μM $FeSO_4$, allowing 20 min incubation at 37°C. Then, 1 ml of 0.6% aqueous solution of TBA and 1 ml of 2.5% trichloroacetic acid were added to each ml of reaction mixture. After heating at 95°C for 30 min and cooling with tap water, the chromogen was extracted with

n-butanol, followed by a brief centrifugation to separate the phases; the organic layer was read at 532 nm against an appropriate TBA-reacted blank. Values were expressed as nmol TBA reactants (TBAR)/ml, using for calculations a molar extinction coefficient of $1.54 \times 10^5\ M^{-1}\ cm^{-1}$. It should be noted that zinc (II) has no effect on the TBA assay per se, as also previously reported.[7,8] For control experiments, the zinc that should have been bound to DMP was omitted; thus, tubes contained 22 μM DMP, pre-incubated for 5 min with 200 μM HOCl, and 4 mM linolenic acid in 0.18 M phosphate buffered saline, pH 7.4; peroxidative reactions were induced by 11 μM $FeSO_4$.

For deoxyribose oxidation, to the DMP (22 μM)-zinc (11 μM) complex, pre-incubated for 5 min at 37°C with 200 μM HOCl in 10 mM KH_2PO_4-KOH buffer, pH 7.4, deoxyribose was added at 8 mM final concentration. Sugar oxidative degradation was induced by 11 μM $FeSO_4$ plus 30 μM H_2O_2, followed by 45 min incubation at 37°C. TBAR were then assessed as previously described for the linolenic acid peroxidation, except that the pink chromogen was not extracted with n-butanol; results were expressed as nmol TBAR/ml. As for iron-dependent linolenic acid oxidation, control experiments were zinc-free.

Other experiments were specifically performed to assess the effects of the zinc (11 μM)-DMP (22 μM) complex, as well as of DMP, on iron-dependent biomolecules oxidant damage. The controls of these experiments were represented by 4 mM linolenic acid in 0.18 M phosphate buffered saline, pH 7.4, and 8 mM deoxyribose in 10 mM KH_2PO_4-KOH buffer, pH 7.4, oxidized by 11 μM $FeSO_4$ and 11 μM $FeSO_4$ plus 30 μM H_2O_2, respectively, in the absence of HOCl. Obviously, this latter could not be added in these specific reaction mixtures to avoid zinc mobilization from the DMP-zinc complex as a result of thiolate bonds oxidation.[4]

Statistics. Data were calculated as means ± SD of 5 different experiments for both HOCl-mediated zinc release and biomolecules oxidation study. Amounts of zinc mobilized from the DMP-zinc complex were analyzed by one-way analysis of variance, followed by the Bonferroni's test to detect significantly different means.[9] Results of the other biochemical tests were computed by the Student's t test for unpaired data. $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

As expected,[4] at 200 μM concentration HOCl induced the mobilization of virtually all the zinc bound to DMP after 5 min incubation (i.e., 10.85 ± 0.17 μM); 100 and 50 μM HOCl released about 60% (6.38 ± 0.25 μM) and 30% (3.4 ± 0.18 μM) of zinc after the same experimental time (200 μM HOCl vs 100 and 50 and μM HOCl, $P < 0.0001$; 100 μM HOCl vs 50 μM HOCl, $P < 0.0001$). According to Fliss and Ménard,[4] this phenomenon was specifically mediated by HOCl, since PAR alone was ineffective to induce metal mobilization from the complex. These Authors have hypothesized that the zinc specifically released from metalloproteins by HOCl may induce cell injury;[4] on the other hand, there is evidence that zinc can be protective via antioxidant mechanisms, with inhibitory effects on transition metal-driven lipid peroxidation.[5] Accordingly, as shown in Table I, the zinc mobilized from thiolate bonds by HOCl induced a significant protection towards the iron-mediated oxidation of both linolenic acid and deoxyribose, which may reflect the capacity of zinc to compete for the oxidizing iron (II) at a variety of biological binding sites (where

TABLE I

Effects of the dimercaptopropanol (DMP)-zinc complex and of the "free" zinc released from the complex by 200 μM HOCl on iron-driven linolenic acid and deoxyribose oxidation

	LINOLENIC ACID	DEOXYRIBOSE
CONTROL 1 (Iron or iron-H_2O_2)	1.1 ± 0.1	0.55 ± 0.08
CONTROL 1 PLUS THE DMP/ZINC COMPLEX	7.6 ± 0.5*	3.55 ● 0.55*
CONTROL 2 (DMP, HOCl, iron or iron-H_2O_2)	9.3 ±0.6	4.35 ± 0.35
"FREE" ZINC (DMP/zinc complex, HOCl, iron or iron-H_2O_2)	5.95 ± 0.4**	3.6 ± 0.25†

Oxidation of 4 mM linolenic acid in 0.18 M phosphate buffered saline, pH 7.4, and of 8 mM deoxyribose in 10 mM potassium phosphate buffer, pH 7.4, was induced by 11 μM $FeSO_4$, and 11 μM $FeSO_4$ plus 30 μM H_2O_2, respectively, followed by the TBA test and in experimental conditions fully described in the MATERIALS and METHODS section. Results represent means ± SD of 5 different experiments, and they are expressed as nmol TBAR/ml.

*, $P < 0.0001$ vs Control 1; **, $P < 0.0001$ vs Control 2; †, $P < 0.01$ vs Control 2.

iron-related radicals are produced in a site-specific manner[10,11]) through a so called "push mechanism".[12] It is noteworthy that the DMP-zinc complex per se showed prooxidant effects on both iron-driven linolenic acid and deoxyribose oxidation (Table I). This phenomenon was apparently due to the sulfhydryl compound DMP; indeed, thiol molecules are capable of increasing iron-dependent oxidant injury.[13] Interconversion of iron between its redox states[13] and, possibly, "malignant" iron chelation with enhanced metal reactivity in Fenton chemistry reactions[14] seem to be involved in thiol-dependent oxidative stress. In such a context, DMP alone also significantly increased iron-mediated oxidation of linolenic acid (7.05 ± 0.35 vs 1.1 ± 0.1 nmol TBAR/ml, $P < 0.0001$) and deoxyribose (2.95 ± 0.3 vs 0.55 ± 0.08 nmol TBAR/ml, $P < 0.0001$). When HOCl was present in specific control reaction mixtures containing DMP and iron, it further stimulated biomolecules oxidant damage, as a consequence of its known prooxidant capacity[1] (Table I). However, when HOCl interacted with the DMP-zinc complex, so that zinc was "free" in solution, a significant antioxidant action of zinc became manifest (Table I). Thus, it is the "free" zinc to possess significant antioxidant properties. This agrees with a previous paper of Thomas *et al.*,[15] demonstrating that the antioxidant capacity of the zinc-binding protein metallothionein is due right to the "free" form of zinc and not to the protein thiol bound one. In our experimental conditions, zinc antagonized more efficiently linolenic acid peroxidation than deoxyribose oxidation, which were inhibited by about 35% ($P < 0.0001$) and 15% ($P < 0.01$), respectively. This phenomenon could be due to a specific interaction of zinc with iron and double bonds of polyunsaturated fatty acids. In such a context, zinc, a redox benign metal with a ligand chemistry similar to that of iron,[12] could inhibit the formation of iron-oxygen-enoic acid complexes (which have been proposed to initiate lipid peroxidation) via a specific Fe^{2+} displacing effect.[7,16,17] Moreover, it has been suggested that zinc antagonized the iron-dependent hydroperoxide breakdown to produce lipoperoxidation-propagating species and secondary lipid oxidation aldehydes, by a specific iron competition for binding to oxygen ligands in oxidized polyunsaturated fatty acids.[8,18]

In conclusion, the zinc released from thiolate bonds by HOCl exerts a significant antioxidant effect towards iron-mediated biomolecules oxidant damage. Zinc concentrations in human plasma are around 15 μM,[19] but they can reach much higher levels, i.e. about 200 μM, in human cells, such as erythrocytes;[20] however, most of zinc is bound to proteins *in vivo*.[19] We have here used a DMP-zinc complex as a recognized model system for zinc mobilization from metalloprotein complexes by HOCl produced by activated neutrophils.[4] In light of the finding that 200 μM HOCl can mobilize all the zinc bound to DMP sulfhydryls and of the high rate of HOCl production by neutrophils at inflamed sites (i.e., even in the millimolar range[21]), localized "free" zinc concentrations in the low micromolar range may be expected in such inflammatory areas, thus suggesting the feasibility of the specific zinc antioxidant mechanisms shown in the present study in the *in vivo* setting. In this regard, both superoxide anion and HOCl, besides H_2O_2, are produced by activated neutrophils;[1] superoxide can induce iron mobilization from ferritin,[22,23] thus triggering oxidative stress.[23] On the other hand, our data indicate that the zinc specifically mobilized from thiolate bonds by HOCl can counteract iron-dependent oxidant damage, thus potentially limiting the cell injury associated with neutrophil activation at inflammatory and/or ischemic-reperfused sites.

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