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Hemin inhibits lipid peroxidation induced by ascorbate/ $FeSO_4$ and 2,2'-azobis-2-amidino-propane hydrochloride (ABAP)

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Abbreviations: MCO, metal-catalyzed oxidation; ABAP, 2,2'-azobis-2-amidino-propane hydrochloride; TBA, thiobarbituric acid; MDA, malondialdehyde

Abstract

Hemin blocked lipid peroxidations induced by either ascorbate/$FeSO_4$, a metal-catalyzed oxidation system, or 2,2'-azobis-2-amidino-propane hydrochloride (ABAP) which produces peroxy radicals at constant rates. Hemin at very low micromolar concentrations strongly inhibited the ascorbate/$FeSO_4$-induced peroxidation of rat liver phopholipids, soybean phosphatidylcholine and arachidonic acid, and this inhibition was also evident with the use of ABAP, although much higher concentrations of hemin were required than those for the inhibition of ascorbate/ $FeSO_4$-induced lipid peroxidation. However, hemoproteins such as hemoglobin, myoglobin and cytochrome C did not show any significant effect on this lipid peroxidation. Hemopexin and albumin abolished the inhibitory action of hemin. During incubation with ascorbate/$FeSO_4$ or ABAP, hemin underwent a change in its absorption spectrum, resulting in a progressive decrease in the peak height of the characteristic absorption band at 385 nm. The above results suggest that hemin may act as an important antioxidant in vivo, protecting lipids from the peroxidative damage.

Keywords: antioxidant, hemin, lipid peroxidation, oxygen free radical

Introduction

Heme (ferroprotoporphyrin IX) compounds are widely distributed in the mammalian tissues, and studies on their roles in the free radical-mediated reactions have been an area of active research (Aft and Muller, 1984a, b; Liu *et al.*, 1985; Gutteridge, 1987; Gutteridge and Smith, 1988; Watts *et al.*, 1995; Miller *et al*., 1996). They have been suggested to act primarily as a prooxidant *in vivo* which can promote the production of oxygen radicals and thus cause an oxidative damage to biomolecules. Heme and hemoproteins have been reported to promote lipid peroxidation in the presence or absence of H_2O_2 and other oxidants (Tappel, 1955; Gutteridge and Smith, 1988; Miller *et al.*, 1996), catalyze the breakdown of lipid hydroperoxides to thiobarbituric acid-reactive products, and cause oxidative modifications of proteins and DNA in the presence of reducing agents (Aft and Muller, 1984a,b; Liu *et al.*, 1985). Although the number of literature is very limited, there also has been some reports suggesting an antioxidative action of heme compounds. Kim *at al.* (1994) showed that hemoglobin and hematin could protect *Escherichia coli* from oxygen radical-mediated killing caused by xanthine oxidase/acetaldehyde and H_2O_2. It was also shown that myoglobin inhibited the lipid peroxidation and tissue injury caused by Fe^{2+}/Fe^{3+}/ADP in rat renal tubular segment (Zager and Foerder, 1992). And a heme-nonapeptide derived from cytochrome C was reported to inhibit the lipid peroxidation in isolated microsomes (Vodnyanszky *et al*., 1985). Thus, heme compounds are suggested to be able to act in the opposite directions as either a prooxidant or an antioxidant depending on the reaction condition.

In this study, we report that hemin can block lipid peroxidation induced by either ascorbate/$FeSO_4$, a metal-catalyzed oxidation system or a temperature-dependent peroxy radical generator, 2,2'-azobis-2-amidino-propane hydrochloride (ABAP).

Meterials and Methods

Chemicals

Hemin chloride, hemoglobin, myoglobin, cytochrome C, soybean phosphatidylcholine, arachidonic acid, ascorbic acid, and human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.), and 2',2'-azobis (2-amidino-propane) dihydrochloride (ABAP) was from Wako Pure Chemical Industry (Osaka, Japan). Hemin stock solution (1 mM) was prepared by dissolving hemin chloride in 0.1 ml of 0.1 M NaOH and adding 0.9 ml H_2O. The concentration of hemin was determined

using its molar extinction coefficient at 385 nm (ε_{mM} = 58.4) (Kirschner-Zilber *et al*., 1982). Hemopexin was purified from human blood plasma by affinity chromatography on hemin-agarose followed by ion exchange chromotography as described by Morgan and Smith (1984).

Isolation of rat liver phopholipids

Phospholids were purified from rat (Sprague-Dawley) liver by the method of Folch *et al.* (1957) with some modifications. The rat liver was minced and homogenized with a Polytron homogenizer in a 12 vol. of chloroform-methanol mixture (2:1, v/v). The homogenate was filtered through a fat-free paper and mixed thoroughly with a 0.5 vol. of KCl solution, and the mixture was allowed to separate into two phases. The upper phase was removed with an aspirator, and the lower phase was dried completely with a rotary vacuum evaporator at 45˚C under the nitrogen phase. Saturated $MgCl_2$ (10 vol. of the lower phase) was added to the dried sample and stood overnight at -20˚C. The soluble neutral lipid fraction was removed by centrifugation at 2,000 *g* for 10 min, and the pellet was rewashed with $MgCl_2$-saturated cold acetone. The pellet containing phospholipids was dissolved in chloroform-methanol and stored at -80˚C. The phospholipid concentration was determined as described by Ames (1966).

Preparation of phospholipid liposomes and fatty acid micelles

The phospholipid liposomes were prepared according to the method described by Motoyama *et al*. (1989). Liver phospholipids or soybean phosphatidylcholine dissolved in chloroform-methanol was placed in a flask, and chloroform-methanol was removed using a rotary vacuum evaporator to obtain a thin lipid film on the flask wall. An appropriate amount of 50 mM sodium phosphate (pH 7.4) was added, and the mixture was shaken vigorouly to obtain a milky suspension. The suspension was then sonicated 3 times at 30 watts for 20 s at 4˚C to obtain unilamellar liposomes. The arachidonic acid stock solution of 1,000 mg/ml was prepared in ethanol, and stored at -20˚C under the nitrogen phase. Immediately prior to use, an aliquot of the stock solution was dried with nitrogen, diluted with 50 mM sodium phosphate (pH 7.4) to a concentration of 4 mg fatty acid/ml, and then sonicated 3 times at 30 watts for 5 s at 4˚C to form fatty acid micelles.

Assay of lipid peroxidation

Phospholipid liposomes or fatty acid micelles (1 mg/ml) were incubated with 0.5 mM ascorbic acid/ 10 μM $FeSO_4$ or 20 mM ABAP in 50 mM sodium phosphate (pH 7.4) in the presence or absence of heme compounds in a total volume of 0.5 ml. After incubation, the reaction mixture was mixed with 1 ml of thiobarbituric acid (TBA) reagent containing 15 % trichloroacetic acid, 0.375% TBA and 2.5 M HCl, and heated at 100˚C for 15 min. After the samples were cooled and centrifuged at 1,000 *g* for 15 min, their absorbances at 535 nm were measured. The amount of TBA-reactive products was calculated as the amount of malondialdehyde (MDA) using the molar extinction coefficient of 156 $mM^{-1}cm^{-1}$ (Lacort *et al.*, 1995).

Results

Figure 1 shows the effects of hemin and hemoproteins on the peroxidation of rat liver phospholipids by ascorbate/ $FeSO_4$. Hemin strongly inhibited the phospholipid peroxidation at low micromolar concentrations in a dose-dependent manner, but hemoglobin, myoglobin and cytochrome c did not show any significant effect on phospholipid peroxidation. A similar result was obtained when the effects of hemin and hemoproteins on phospholipid peroxidation were examined using ABAP as an oxidant (Figure 2). Thus, hemin significantly inhibited the ABAP-induced phospholipid peroxidation over a wide range of concentrations, whereas hemoglobin, myoglobin and cytochrome c did not inhibit at all. However, the inhibitory action of hemin was not so potent in the ABAP system as in the ascorbate/$FeSO_4$ system. The concentrations of hemin required to inhibit the ascorbate/$FeSO_4$- and ABAP-induced phospholipid peroxidation by 50% were 1 and 40 μM, respectively. Hemin could also inhibit the ascorbate/$FeSO_4$- and ABAP-induced peroxidation of soybean phosphatidylcholine and arachidonic acid (Figure

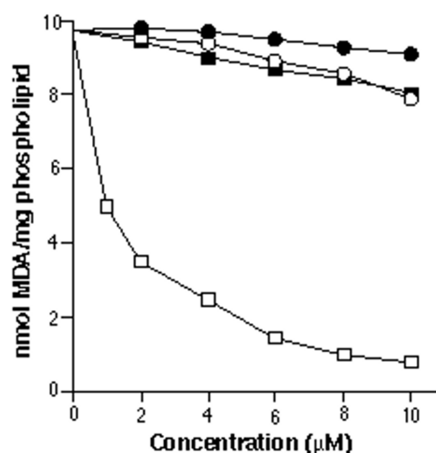


Figure 1. Effects of hemin and hemoproteins on phospholipid peroxidation by ascorbate/$FeSO_4$. Reaction mixture (0.5 ml) containing 1 mg/ml rat liver phospholipids, 0.5 mM ascorbic acid, 10 μM $FeSO_4$, 50 mM sodium phosphate (pH 7.4) and hemin or hemoprotein at the indicated concentrations was incubated at 37˚C for 30 min. ●, Hemoglobin; ○, myoglobin; ■, cytochrome c; □, hemin.

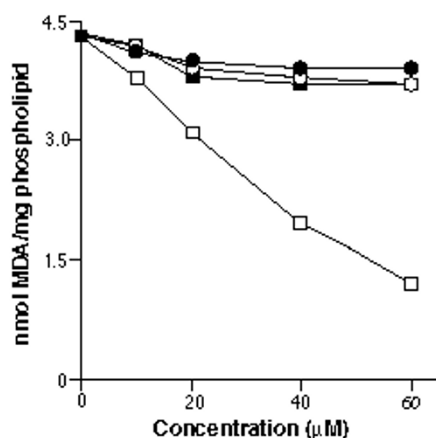


Figure 2. Effects of hemin and hemoproteins on phospholipid peroxidation by ABAP. Reaction mixture (0.5 ml) containing 1 mg/ml rat liver phospholipids, 20 mM ABAP, 50 mM sodium phosphate (pH 7.4), and hemin or hemoprotein at the indicated concentrations was incubated at 37˚C for 30 min. ●, Hemoglobin; ○, myoglobin; ■, cytochrome c; □, Hemin.

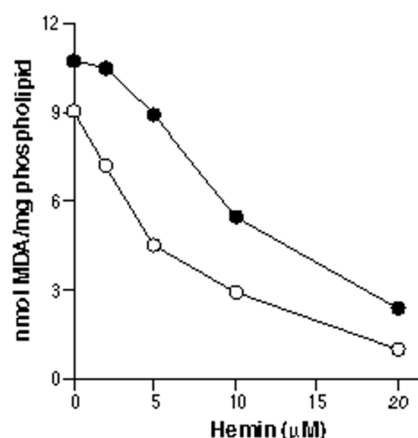


Figure 3. Inhibitory effects of hemin on soybean phosphatidylcholine (SBPC) and arachidonic acid (AA) peroxidation by ascorbate/$FeSO_4$. Reaction mixture (0.5 ml) containing 0.5 mM ascorbic acid, 10 μM $FeSO_4$, 1 mg/ml SBPC or AA, 50 mM sodium phosphate (pH 7.4), and hemin at the indicated concentrations was incubated at 37˚C for 30 min. ●, AA; ○, SBPC.

Table 1. Blocking effects of hemopexin and albumin on the lipid peroxidation-inhibiting action of hemin. Reaction mixture (0.5 ml) containing 1 mg/ml phopholipids, 0.5 mM ascorbic acid, 10 μM $FeSO_4$, 50 mM sodium phophate (pH 7) and additives as indicated was incubated at 37˚C for 30 min.

Additives	Lipid peroxidation	
	nmol MDA/mg[a] phospholipid	(%)
None	9.83 ± 0.51	100
Hemopexin (5 μM)	9.78 ± 0.48	99.5
Albumin (5 μM)	9.75 ± 0.59	99.2
Hemin (5 μM)	1.76 ± 0.13	17.9
Hemin (5 μM) + albumin (5 μM)	4.18 ± 0.32	42.5
Hemin (5 μM) + hemopexin (5 μM)	7.91 ± 0.38	80.5

[a] The data represent the mean ± S.D. from triplicate experiments.

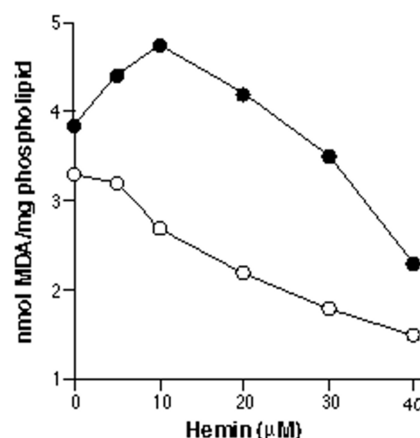


Figure 4. Inhibitory effects of hemin on soybean phosphatidylcholine (SBPC) and arachidonic acid (AA) peroxidation by ABAP. Reaction mixture (0.5 ml) containing 20 mM ABAP, 1 mg/ml SBPC or AA, 50 mM sodium phosphate (pH 7.4), and hemin at the indicated concentrations was incubated at 37˚C for 30 min. ●, AA; ○, SBPC.

3 and 4). Hemin inhibited the ascorbate/$FeSO_4$-induced peroxidation of phosphatidylcholine and arachidonic acid at micromolar concentrations as strongly as it did the ascorbate/$FeSO_4$-induced peroxidation of rat liver phospholipids. However, dose-dependent effects of hemin on ABAP-induced peroxidation of arachidonic acid showed peculiar profiles in which hemin acted biphasically: it rather promoted the fatty acid peroxidation slightly at concentrations less than 10 μM, but at concentrations above 10 μM it inhibited the fatty acid peroxidation in a dose-dependent manner. This result is reminescent of earlier observations (Vincent *et al*., 1988; Schmitt *et al.*, 1993) in which lipid peroxidation was shown to display a bell-shaped dependency on the hemin concentration: the extent of lipid peroxidation increased progressively as the hemin concentration increased to a certain point but it decreased progressively as the hemin concentration increased beyond the point.

Table 1 shows the effects of hemopexin and HSA, heme-binding proteins (Vincent *et al*., 1988), on the inhibitory action of hemin. Hemopexin and albumin markedly blocked the inhibitory action of hemin during the phospholipid peroxidation caused by ascorbate/$FeSO_4$ and ABAP. This result supports the above observations,

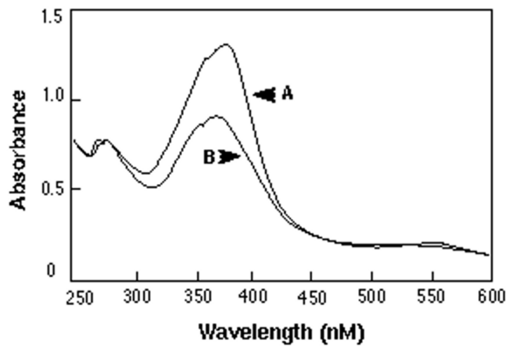


Figure 5. Change in the absorption spectrum of hemin following incubation with ABAP. 20 μM hemin was incubated with 20 mM ABAP in 50 mM sodium phosphate (pH 7.4) at 37˚C for 1 h. A, Before incubation B, After incubation.

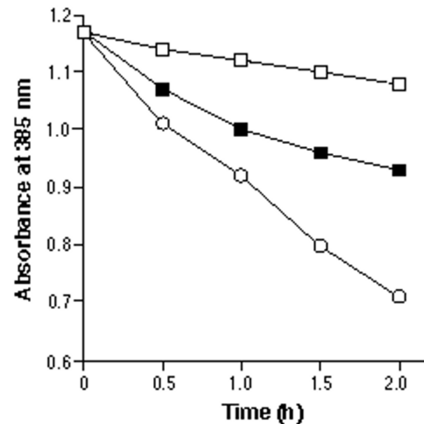


Figure 6. Changes in absorbance at 385 nm of hemin during incubation with ascorbate/$FeSO_4$ and ABAP. Reaction mixture (1 ml) containing 20 μM hemin, 50 mM sodium phosphate (pH 7.4), and 0.5 mM ascorbic acid/10 μM $FeSO_4$ or 20 mM ABAP was incubated at 37˚C for the indicated time. ○, Control; ■, ascorbate/$FeSO_4$; □, ABAP.

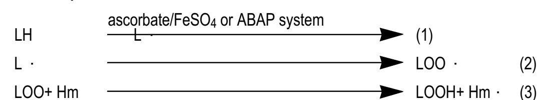
indicating that hemin can inhibit the lipid peroxidation caused by ascorbate/$FeSO_4$ and ABAP systems.

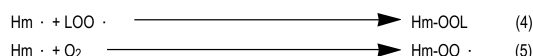
During the incubation of hemin with ascorbate/$FeSO_4$ or ABAP, the absorption spectrum of hemin changed. As shown in Figure 5, the height of the characteristic absorption peak of hemin around 385 nm was decreased by the oxidative challenge. Figure 6 shows that the absorbance at 385 nm of reaction mixtures decreased linearly with time during the incubation of hemin with the above oxidation systems. These results suggest that hemin is modified by the oxidation systems and that hemin may inhibit the lipid peroxidation possibily through acting as a sacrificial antioxidant.

Discussion

There have been a number of reports describing that oxidative damage of biological molecules can be mediated by hemoglobin, myoglobin and hemin (Aft and Muller, 1984a,b; Sadrzadeh *et al*., 1984, 1987; Puppo and Halliwell, 1988; Fantone *et al*., 1989; Galaris *et al*., 1990; Schmitt *et al*., 1993). It is suggested that in these reactions the heme group may either act as a Fenton reagent which can produce hydroxyl radicals or produce oxidizing species other than the hydroxyl radical such as ferryl intermediates and radical species in amino acid residues of hemoproteins. In contrast, there has been few literature describing the antioxidative action of heme compounds (Vodnyanszky *et al*., 1985; Galaris *et al*., 1989; Kim *et al*., 1994). Our present results clearly show that hemin can inhibit the lipid peroxidation induced by either ascorbate/$FeSO_4$, a metal-catalyzed oxidation system, or ABAP which produces peroxy radicals. Although hemoproteins such as hemoglobin, myoglobin and cytochrome c did not show any significant effect on the lipid peroxidation by the above oxidation systems, hemin could inhibit the ascorbate/$FeSO_4$-induced lipid peroxidation at very low micromolar concentrations. These results suggest that hemin may act as an important antioxidant in the physiological processes in which ascorbate/$FeSO_4$ and other metal-catalyzed oxidation systems are involved.

According to Kim *et al*. (1994), when *E. coli* was incubated with xanthine oxidase/acetaldehyde or H_2O_2, the killing of *E. coli* was accelerated by iron-EDTA, but inhibited by hematin or hemoglobin. They suggested that hematin and hemoglobin decompose H_2O_2 to other oxidizing species to which bacteria are not susceptible. Zager and Foerder (1992) reported that myoglobin could protect against iron-mediated renal tubular lipid peroxidation and cytotoxicity, and suggested that residual proteins resulting from the liberation of iron from the porphyrin ring may serve to mitigate the iron-mediated damage. However, our present study shows quite different results. Hemin inhibited not only the lipid peroxidation caused by the ascorbate/$FeSO_4$ system, but also that caused by the ABAP system in which H_2O_2 is not involved in the lipid peroxidation. In addition, unlike hemin, hemoproteins such as hemoglobin and myoglobin did not inhibit the lipid peroxidation by ascorbate/ $FeSO_4$ and ABAP. Therefore, other mechanism(s) different from those mentioned above seems to be involved in the action of hemin observed in this study. Although the exact mechanism is not known, some feasible mechanisms can be speculated. First, hemin (Hm) may react with peroxy radicals (LOO ·) derived from unsaturated fatty acids (LH) to give rise to non-radical or less reactive radical products as follows.





Hemin contains conjugated double bonds, which may react with peroxy radicals to form resonance-stabilized radicals. Second, the lipid peroxidation-inhibiting action of hemin may be attributed to the higher reactivity of hemin to oxygen radicals than that of unsaturated fatty acids. Schacter *et al*. (1972) reported that during microsomal lipid peroxidation microsomal heme degradation occurred rapidly with the fission of one methene bridge in the tetrapyrrole ring. As shown in Figure 6, the incubation of hemin with the ascorbate/ $FeSO_4$ or ABAP system resulted in a decrease in the absorption peak of hemin at the 385 nm, indicating that hemin was modified during incubation with the oxidation systems. These results suggest, therefore, that hemin may act as a sacrificial antioxidant against the oxidative challenge. Finally, the fact that hemin inhibited the ascorbate/$FeSO_4$-induced lipid peroxidation more strongly than the ABAP-induced lipid peroxidation suggests that another mechanism by which H_2O_2, •OH or $•O_2^-$ can be removed more readily than can the peroxy radical may also be involved in the hemin action. Hemin may directly decompose H_2O_2 or scavenge the hydroxyl radical. Further studies are required to verify these assumptions.

Acknowledgement

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