

Lipid Peroxidation Induced by the Reaction of Cytochrome *c* with Hydrogen Peroxide

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Lipid peroxidation induced by the reaction of cytochrome *c* with H₂O₂ was investigated. When linoleic acid micelles or phosphatidyl choline liposomes were incubated with cytochrome *c* and H₂O₂, lipid peroxidation was increased in cytochrome *c* and H₂O₂ concentrations-dependent manner. Radical scavengers, azide, formate and ethanol prevented lipid peroxidation induced by the cytochrome *c*/H₂O₂ system. Iron specific chelator, desferoxamine also prevented the cytochrome *c*/H₂O₂ system-mediated lipid peroxidation. These results suggest that lipid peroxidation may be induced by the cytochrome *c*/H₂O₂ system via the generation of free radicals. Carnosine, homocarnosine and anserine are present in the muscle and brain of many animals and human. Previous studies show that these compounds have an antioxidant function. In the present study, carnosine, homocarnosine and anserine significantly prevented the cytochrome *c*/H₂O₂ system-mediated lipid peroxidation. Carnosine and related compounds also inhibited the free radical-generating activity of cytochrome *c*. The results suggest that carnosine, homocarnosine and anserine may prevent lipid peroxidation induced by the cytochrome *c*/H₂O₂ system through a free radical scavenging.

Key Words : Cytochrome *c*, Lipid peroxidation, Free radical, Carnosine

Introduction

Cytochrome *c* is an electron transport protein and it catalyses peroxidase-like reactions *in vitro*.¹ It catalyses several reactions such as hydroxylation and aromatic oxidation and shows peroxidase activity by oxidation of various electron donors such as 2-keto-4-thiomethyl butyric acid, and 4-aminoantipyrine.² Recently, it has been reported that this protein was closely related with two other biological processes, apoptosis and oxidative stress.^{3,4} Apoptosis, also called programmed cell death, is key to development and is linked to human diseases, including cancer and neurodegeneration.^{5,6} The cellular damage associated with oxidative stress has also been related with several diseases, such as Parkinson's disease (PD).⁷ One consequence of the long exposure to oxidants is the oxidation of lipids (called lipid peroxidation) in cell membranes and lipoproteins. It is becoming increasingly evident that peroxidative damage to low-density lipoprotein plays a key role in the etiology of atherosclerosis.^{8,9} Lipid peroxidation may also contribute to other human diseases, such as certain types of cancer, rheumatoid arthritis, and myocardial reoxygenation injury as well as to the degenerative processes associated with aging.¹⁰ Although the ability of the cytochrome *c* and H₂O₂ system to oxidize organic molecules is well known, a mechanism for the lipid peroxidation by this system has not been reported.

In this study, the cytochrome *c* and H₂O₂ system-mediated lipid peroxidation was studied. The present results suggest that the lipid peroxidation induced by cytochrome *c* and H₂O₂ is due to the oxidative damage resulting from free radicals generated by a combination of the peroxidase activity of cytochrome *c* and the Fenton reaction of free iron released from oxidatively damaged cytochrome *c*.

Materials and Methods

Materials. Desferoxamine (DFX), linoleic acid, soybean phosphatidylcholine, thiobarbituric acid and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA, U.S.A.).

Preparation of phospholipid liposomes and fatty acid micelles. The phospholipid liposomes were prepared according to the method previously described.¹¹ Soybean phosphatidylcholine dissolved in chloroform-methanol was placed in a round bottom 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 10 mM potassium phosphate (pH 7.4) was added, and the mixture was shaken vigorously to yield a milky suspension. The suspension was then sonicated 3 times at 30 watts for 20 s at 4 °C to obtain unilamellar liposomes. The linoleic acid stock solution of 10 mg/mL was prepared in ethanol, and stored at -20 °C under nitrogen. Immediately prior to use, an aliquot of the stock solution was dried with nitrogen, diluted with 10 mM potassium phosphate (pH 7.4) to a concentration of 10 mg fatty acid/mL, and then sonicated 3 times at 30 watts for 5 s at 4 °C to form fatty acid micelles.

Assay for lipid peroxidation. As an index of lipid peroxidation, thiobarbituric acid-reactive substance (TBARS) was determined by a modification of the method previously described.¹² Linoleic acid micelles (1 mg/mL) or phosphatidylcholine liposomes (1 mg/mL) were incubated with different concentrations of cytochrome *c* and H₂O₂ in 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 100 μ L. The incubations were performed at 37 °C for various periods. The reaction was stopped by the addition of 200 μ L of 6% trichloroacetic acid. The reaction mixture was

mixed with phosphate buffered saline (200 μL), and 1% thiobarbituric acid (200 μL), and boiled at 100 $^{\circ}\text{C}$ for 15 min. After the samples were cooled and centrifuged at 15,000 rpm for 10 min. The differential absorbance of the supernatant fractions at 532 nm was measured spectrophotometrically. The amount of TBARS was determined by comparison to a standard curve constructed using malondialdehyde (MDA). All solutions were treated with Chelex 100 resin to remove any traces of transition metal ions.

Measurement of free radical. The free radical-generating activity of cytochrome *c* was measured by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS).¹³ ABTS is water-soluble and has a strong absorption at 340 nm with a molar extinction coefficient ϵ_{340} of $3.66 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.¹⁴ On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical, $\text{ABTS}^{+\cdot}$ is conveniently followed at λ_{max} at 415 nm ($\epsilon_{415} = 3.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$).¹⁵ The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4) and 50 μM ABTS and 0.3 mM hydrogen peroxide and 5 μM protein in a total volume of 1 mL. The reaction was initiated by addition of hydrogen peroxide and the increase in absorbance at 415 nm was measured by using a UV/Vis spectrophotometer (Shimadzu 1601).

Replicates. Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments

Results and Discussion

Figure 1 shows the peroxidation of fatty acid and phospholipid induced by the cytochrome *c*/ H_2O_2 system. The peroxidation of linoleic acid micelles did not occur after the incubation with 1.5 μM cytochrome *c* or 150 μM H_2O_2 alone. However, when the fatty acid micelles were incubated in a mixture of cytochrome *c* and H_2O_2 , lipid peroxidation was increased for 120 min in a time-dependent manner (Fig. 1A). A similar result was obtained when the phosphatidylcholine liposomes were incubated in the cytochrome *c*/ H_2O_2 system (Fig. 1B). These results indicate that both cytochrome *c* and H_2O_2 were required for lipid peroxidation. The time course of the reaction for the generation of the peroxidized lipid products, measured with thiobarbituric acid-reactive substance (TBARS), in linoleic acid micelles was more rapid and pronounced than in the phosphatidylcholine liposomes. This may be because the liberated linoleic acid has a relatively greater propensity for free radical attack than the variety of polyunsaturated fatty acids present in the phospholipid bilayer of the biomembranes. The effects of cytochrome *c* and H_2O_2 concentrations on lipid peroxidation were studied. The peroxidation of both the linoleic acid micelles and phosphatidylcholine liposomes was increased by concentrations up to 3 μM cytochrome *c* (Fig. 2A) and 300 μM H_2O_2 (Fig. 2B), respectively. The results show that lipid peroxidation induced by the cytochrome *c*/ H_2O_2 system is dependent on the concentrations of cytochrome *c* and H_2O_2 .

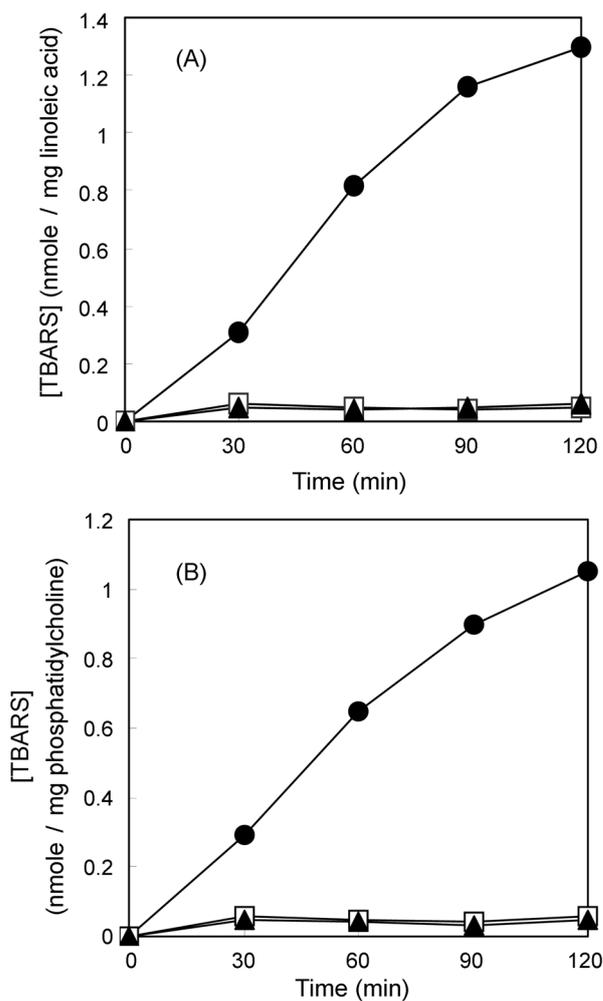


Figure 1. Lipid peroxidation induced by the incubation with cytochrome *c* and H_2O_2 . Linoleic acid micelles (A) or phosphatidylcholine liposome (B) was incubated at 37 $^{\circ}\text{C}$ for various incubation periods with the following; 1.5 μM cytochrome *c* alone (\square); 150 μM H_2O_2 alone (\blacktriangle); 1.5 μM cytochrome *c* plus 150 μM H_2O_2 (\bullet).

Previous reports showed that free radicals were generated in the reaction of cytochrome *c* with H_2O_2 , and that free radical formation was linear with respect to the concentrations of cytochrome *c* and H_2O_2 .¹³ The participation of free radical in lipid peroxidation induced by the cytochrome *c*/ H_2O_2 system was studied by examining the protective effects of the free radical scavengers such as azide, formate, and ethanol. When the linoleic acid micelles or the phosphatidylcholine liposomes were incubated with cytochrome *c* and H_2O_2 in the presence of radical scavengers, all scavengers significantly prevented lipid peroxidation (Table 1). These results suggest that free radicals may participate in the mechanism of lipid peroxidation induced by the cytochrome *c*/ H_2O_2 system.

Trace metal such as iron and copper, which are variously present in biological systems, may interact with reactive oxygen species, to damage macromolecules.¹⁶⁻¹⁹ The cleavage of the metalloproteins by oxidative damage may lead to

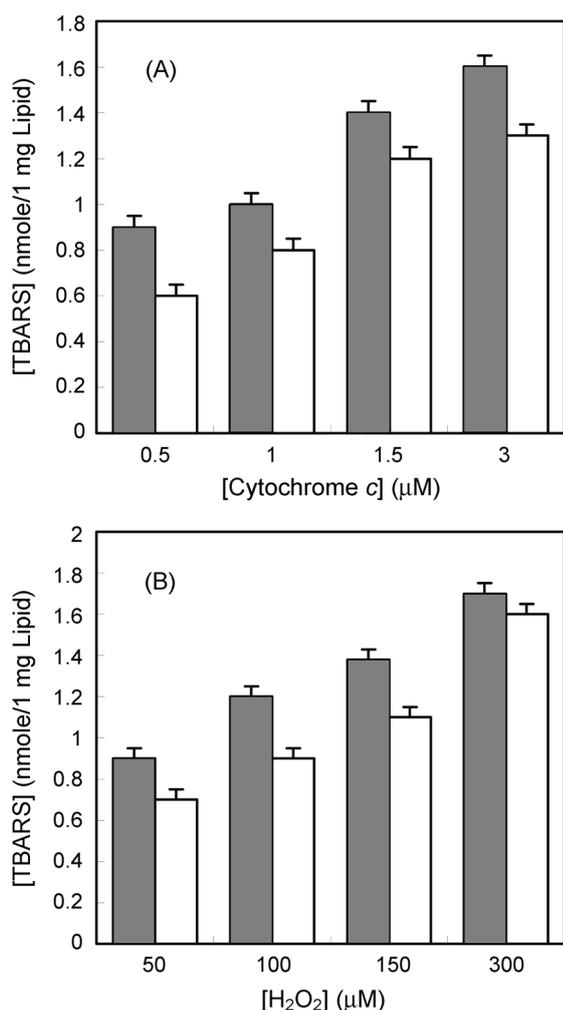


Figure 2. Effects of cytochrome *c* and H₂O₂ concentrations on the lipid peroxidation. (A) Linoleic acid micelles (■) or phosphatidylcholine liposome (□) was incubated with 0.5–3 μM cytochrome *c* and 150 μM H₂O₂ at 37 °C for 2 h. (B) Linoleic acid micelles (■) or phosphatidylcholine liposome (□) was incubated with 1.5 μM cytochrome *c* and 50–300 μM H₂O₂.

increases in the levels of metal ions in some biological cell.²⁰ Previous reports showed that a metal chelator inhibited the peroxidase activity of cytochrome *c*.²¹ The participation of iron ions in the cytochrome *c*/H₂O₂-mediated lipid peroxidation was investigated by an examination of the protective effects of the iron chelator DFX. The results showed that DFX prevented the peroxidation of both the linoleic acid micelles and phosphatidylcholine liposomes induced by the cytochrome *c*/H₂O₂ system (Fig. 3). It has been reported that iron ions released from the reaction of cytochrome *c* with H₂O₂. This is likely due to the peroxidase activity of cytochrome *c*. Since iron ions could stimulate Fenton reaction to produce hydroxyl radical, lipid peroxidation is mediated in the cytochrome *c*/H₂O₂ system *via* the generation of hydroxyl radicals.

The previous reports showed that the interaction between ferricytochrome *c* and hydrogen peroxide would initially from an oxoferryl derivative of hemoprotein.¹³ Oxoferryl

Table 1. Effect of radical scavengers on lipid peroxidation induced by the cytochrome *c* and hydrogen peroxide system

Additive	Concentration	Linoleic acid		Phosphatidylcholine	
		Amount of TBARS (nmole/mg Lipid) (%)	Amount of TBARS (%)	Amount of TBARS (nmole/mg Lipid) (%)	Amount of TBARS (%)
None		1.46 ± 0.06	100	1.17 ± 0.04	100
Azide	10 mM	0.48 ± 0.05	33	0.36 ± 0.06	30
	50 mM	0.02 ± 0.05	1	0.04 ± 0.06	4
Formate	10 mM	0.68 ± 0.02	47	0.52 ± 0.03	45
	50 mM	0.04 ± 0.03	3	0.05 ± 0.03	4
Ethanol	10 mM	0.87 ± 0.05	60	0.82 ± 0.06	70
	50 mM	0.26 ± 0.07	18	0.31 ± 0.03	26

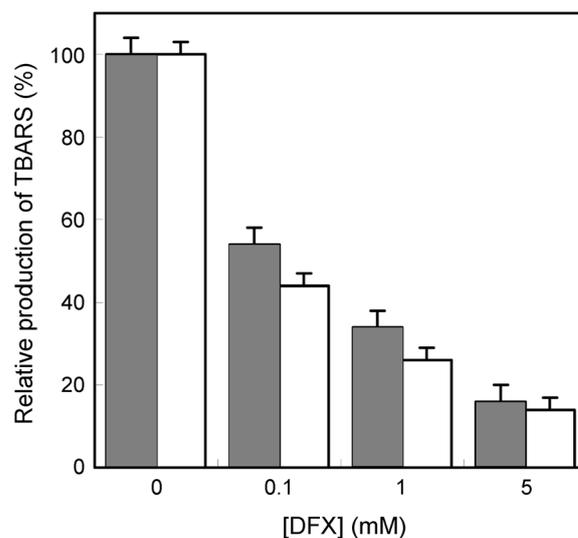


Figure 3. Effect of DFX on the lipid peroxidation induced by the cytochrome *c* and H₂O₂. Linoleic acid micelles (■) or phosphatidylcholine liposomes (□) was incubated with 1.5 μM cytochrome *c* and 150 μM H₂O₂ at 37 °C for 2 h. in the absence of DFX or in the presence of various concentrations of DFX.

cytochrome *c*, similar to compound I of peroxidases, is assumed to contain two oxidizing equivalents, one in the form of oxoferryl heme (Fe^{IV}=O), and another as the porphyrin π radical.¹³ Recent study reported that melatonin oxidation by cytochrome *c* would be mediated through the family of oxoferryl heme species of cytochrome *c*.²² Hydrogen peroxide could induce a short-lived cytochrome *c*-derived tyrosyl radical as detected by the electron spin resonance spin-trapping technique.²³ Therefore, the present results suggest that lipid peroxidation can be mediated by an oxoferryl heme species or the protein tyrosyl radical as well as a Fenton-type reaction producing hydroxyl radical. Evidence that the iron chelator DFX and hydroxyl radical scavengers could not completely prevent the reaction activity of cytochrome *c* supports this mechanism.

Carnosine and related compounds, homocarnosine, anserine are present in the muscle and brain tissues of human and other vertebrates in relatively high concentrations (1–20 mM).^{24,25} These compounds have been shown to be very efficient antioxidant molecules.²⁶ It was found that carnosine

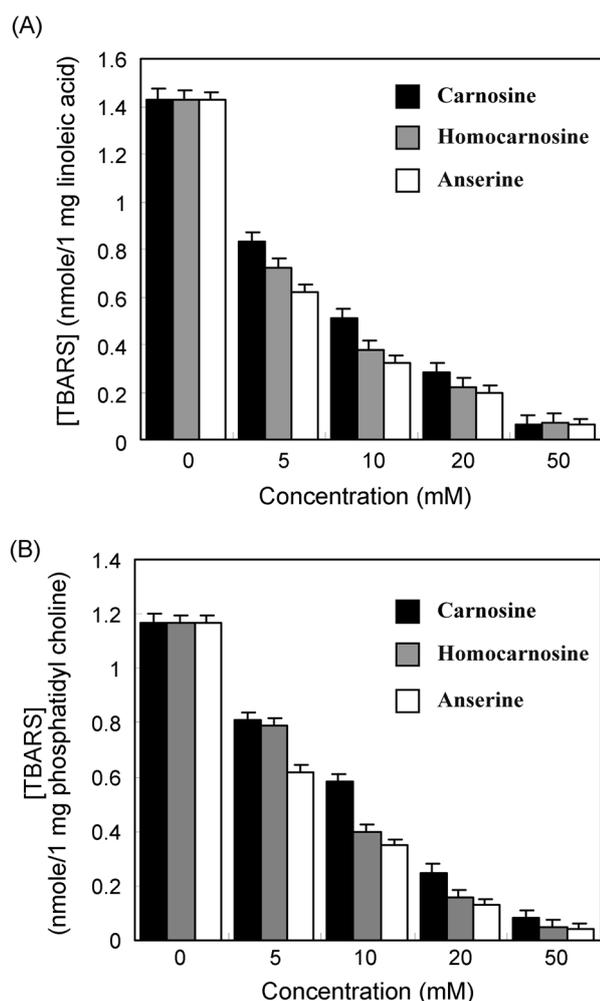


Figure 4. Effects of carnosine and related compound on the lipid peroxidation in the cytochrome *c* and H_2O_2 system. Linoleic acid micelles (A) or phosphatidylcholine liposome (B) was incubated with $1.5 \mu M$ cytochrome *c* and $150 \mu M$ H_2O_2 at $37^\circ C$ for 2 h in the presence of various concentrations of carnosine (■), homocarnosine (■) and anserine (□).

and related compounds significantly inhibited the lipid peroxidation induced by the cytochrome *c*/ H_2O_2 system (Fig. 4). It has been reported that carnosine and related compounds quench 50-95% of hydroxyl radicals produced in the Fenton reaction.²⁷ The effects of carnosine and related compounds on the generation of free radical by the cytochrome *c*/ H_2O_2 system were examined. The free radical-generating activity of cytochrome *c* was measured by using a chromogen, 2,2'-azino-bis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS).¹³ ABTS is water-soluble and has a strong absorption at 340 nm with a molar extinction coefficient ϵ_{340} of $3.66 \times 10^4 M^{-1} cm^{-1}$.²¹ Attack of $\cdot OH$ on ABTS forms a stable blue-green product presumed to be the cation radical, $ABTS^{+\cdot}$ is conveniently followed at λ_{max} at 415 nm.¹⁴ This can be used to detect $\cdot OH$ production, although it is unclear whether or not some other reactive oxygen species can also form $ABTS^{+\cdot}$. When cytochrome *c* was incubated with H_2O_2 in the presence of carnosine, homocarnosine and anserine at

Table 2. Effect of carnosine and related compounds on the formation of $ABTS^{+\cdot}$ by cytochrome *c* / H_2O_2 system

Additive	Concentration	$ABTS^{+\cdot}$	
		$\mu mole/min$	% of control
None		0.597 ± 0.06	100
Carnosine	0.1 mM	0.292 ± 0.05	49
	1 mM	0.124 ± 0.04	21
Homocarnosine	0.1 mM	0.306 ± 0.08	51
	1 mM	0.167 ± 0.06	28
Anserine	0.1 mM	0.313 ± 0.06	52
	1 mM	0.235 ± 0.05	39

$37^\circ C$, all compounds significantly inhibited the free radical-generating activity of cytochrome *c* (Table 2). These compounds might be able to bind Cu^{2+} and prevent some Cu^{2+} -dependent radical reaction. However, it has not been found to chelate iron in a manner that reduces its prooxidant activity.²⁴ Thus, the ability of carnosine to prevent the cytochrome *c*/ H_2O_2 -mediated lipid peroxidation is likely due to free radical scavenging activity.

Lipid peroxidation has been hypothesized to play a critical role in a number of serious human diseases, such as cancer, atherosclerosis, rheumatoid arthritis and myocardial reoxygenation injury as well as in the degenerative processes associated with aging.^{28,29} It has been reported that at least $0.1 mM$ H_2O_2/min can be produced continuously under physiological conditions and at a much higher rates under adverse conditions such as hyperoxia or ischemia and reperfusion.^{30,31} Hence, under these abnormal conditions, the oxidative damage of lipid induced by the cytochrome *c* system may be operative *in vivo*.

In conclusion, the results presented here suggest that lipid peroxidation can be induced by the reaction of cytochrome *c* with H_2O_2 , involving free radical generation. It can be assumed that carnosine, homocarnosine and anserine may protect the lipid peroxidation the cytochrome *c*/ H_2O_2 system through the mechanism of free radical scavenging.

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