Note

Antioxidative Effect of Human Saliva on Lipid Peroxidation

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Lipid peroxidation has attracted much attention in relation to oxidative damage that leads to a wide variety of pathological conditions. In addition, it has become apparent that the human body possesses a well-organized cellular and extracellular defense system against lipid peroxidation and its related-oxygen radicals. This system involves low molecular weight antioxidants such as tocopherols, ascorbic acid and uric acid.^{1–3)} Human saliva plays an essential role in maintaining the physiological conditions of oral tissues.⁴⁾ The processes for mastication and digestion of ingested foods seem to promote a variety of reactions, including lipid peroxidation. Secretion of this extracellular fluid may also participate in the defense system against lipid peroxidation, because uric acid is known to be present in human saliva.⁵⁾

The purpose of this work is to learn whether human saliva can exert an antioxidative effect of lipid peroxidation that occurs in ingested foods. We examined the effect of parotid saliva on the peroxidation of liposomes of egg yolk phosphatidylcholine (PC) and homogenates from cooked fish. The results strongly suggest that human saliva has a role in suppressing the lipid peroxidation of ingested foods.

Egg yolk PC (99% grade) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PC hydroperoxides (PC-OOH) were prepared by a photosensitized oxidation of egg yolk PC according to the method described previously⁶⁾. 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) was generously provided by Wako Pure Chemicals (Osaka, Japan), and the solvents for chromatography were of HPLC grade from Nakarai Chemicals (Kyoto, Japan). Distilled water was further purified by the Milli-Q reagent system (Millipore Corporation, Bedford, MA, U.S.A.).

Parotid saliva was collected from normal adult volunteers, the salivary flow being stimulated with citric acid troches before collecting directly by Lashery's device.⁷⁾ The collected saliva was immediately used for the experiments.

PC liposomes were prepared from a hexane solution of PC which was evaporated under nitrogen, the dried PC

(6.2 μ mol) being dispersed in 0.5 ml of Tris-HCl buffer (0.01 M pH 7.4). Multilamellar liposomes were obtained by mixing this suspension with a Vortex mixer for 30 sec, and then ultrasonically irradiating in a Branson sonifier for 1 min at 30°C. To this suspension, 0.5 ml of parotid saliva or normal saline was added and mixed in vigorously. Lipid peroxidation was started by adding 10 μ l of an aqueous solution of AAPH (20 mM in final conc.) or ferrous sulfate (0.4 mM in final conc.) and ascorbic acid (4 mM). Incubation was carried out at 37°C with continuous shaking; at regular intervals, aliquots of the sample (20 μ l) were withdrawn and mixed with 20 μ l of methanol. This mixture was then injected into a HPLC column for the determination of PC-OOH, and aliquots of the sample (10 μ l) were withdrawn for the determination of uric acid and ascorbic acid.

PC-OOH was determined by reversed-phase HPLC, using an octane-bonded silica column (YMC-packed-212-C8, 6 $\times$ 150 mm) with UV and electrochemical (EC) detection.⁸⁾ Uric acid and ascorbic acid in the saliva and the reaction mixture were also determined by reversed-phase HPLC, using an octadecane-bonded silica column (YMC-packed 312-ODS) with EC detection according to the method of Iriyama *et al.*⁹⁾ A TBA assay was carried out according to the procedure of Uchiyama and Mihara.¹⁰⁾

Human saliva is excreted from the parotid, submandibular, sublingual and the large number of minor salivary glands in the mouth.⁴⁾ The total amount of excreted saliva has been estimated to be about 600 ml/day/person.⁴⁾ Secretion from the parotid gland can be increased by stimulation, and in this work, parotid saliva was used because of the simplicity of collecting contaminant-free material. The concentrations of ascorbic acid and uric acid in parotid saliva from seven male donors were determined to be $6.0 \pm 5.6 \mu\text{M}$ (0–17.5 μM) and $241 \pm 83 \mu\text{M}$ (123–371 μM), respectively. Although we tried to determine the amount of tocopherols in the parotid saliva, the salivary tocopherol level was lower than the detection limit for the analysis method used¹¹⁾ (< 1.3 nM).

Figure 1A shows AAPH-induced liposomal lipid peroxidation in saline and saliva. A biphasic oxidation curve was observed in the reaction mixture with saliva, while uric acid decreased linearly in the initial stage and PC-OOH accumulated rapidly after the uric acid had disappeared completely. Therefore, uric acid was found to be responsible for the inhibition of PC-OOH accumulation. When the liposomal peroxidation was started by Fe^{2+} and ascorbic acid, PC-OOH was scarcely noticeable in the chromatograms of both reaction mixtures (not shown here). However, thiobarbituric acid reactive substances (TBARS) were produced as incubation proceeded in the mixture with normal saline (Fig. 1B). The amount of TBARS in the mixture with saliva was maintained at a lower level throughout the incubation period, while uric acid scarcely decreased. There is a possibility that the saliva affected the TBA reaction. However, no difference was

Fig. 1. Lipid Peroxidation of the Mixture of a Liposomal Suspension of Egg Yolk PC by AAPH (A) and Fe^{2+} (B).

○, the mixture with normal saline; ●, the mixture with parotid saliva; □, relative % of uric acid in the mixture with parotid saliva. The reaction was initiated by (A) adding of AAPH (20 mM) or (B) FeSO_4 (0.4 mM) and ascorbic acid (4 mM) (B). The initial level of endogenous uric acid and ascorbic acid in the mixture with parotid saliva were $65.5 \mu\text{M}$ and $5.3 \mu\text{M}$, respectively.

Table I. EFFECT OF PAROTID SALIVA ON Fe^{2+} -INDUCED DECOMPOSITION OF PC-OOH

PC-OOH ($0.192 \mu\text{mol}$), FeSO_4 (0.4 mM) and ascorbic acid (4 mM) were added to 0.2 ml of the fluid, which was then incubated for 10 min at 37°C . Triplicate experiments were run, and the mean $\pm$ S.D. are presented.

System	Addition	PC-OOH (%) ^a	PC-OH (%) ^b	PC-OH/PC-OOH
Normal saline	None	85 ± 8	15 ± 5	0.18 ± 0.07
	Fe^{2+} ascorbic acid	34 ± 5	9 ± 5	0.29 ± 0.17
Saliva	None	87 ± 13	15 ± 11	0.20 ± 0.16
	Fe^{2+} ascorbic acid	58 ± 9	10 ± 7	0.18 ± 0.14

^a Determined by EC detection.

^b Calculated by subtracting the amount obtained by EC detection from that obtained by UV detection.

observed between the TBARS value of the mixture with saline ($33.8 \pm 1.7 \text{ nmol/ml}$) and with saliva ($35.7 \pm 2.7 \text{ nmol/ml}$) when they were mixed with a liposomal suspension after incubating the liposomal suspension under the same conditions. Thus, saliva did not suppress the TBA reaction, but did the formation of TBARS by incubating liposomal lipids with Fe^{2+} . Table I shows the effect of saliva on the Fe^{2+} -dependent decomposition of PC-OOH. Our previous study⁸⁾ clarified that the amount monitored by the EC detector is attributable to that of PC-OOH, while PC-OOH and its reduced alcohol (PC-OH) cannot be well resolved by this HPLC technique, although both are detectable by the UV method. Thus, the amounts of PC-OH were tentatively calculated by subtracting the amount obtained by EC detection from that by UV detection. Without Fe^{2+} and ascorbic acid, the recovery of PC-OOH from both saline and saliva was estimated to be more than 80% after incubating for 10 min at 37°C . On the other hand, the recovery from saliva was significantly higher than that from saline when the mixture

containing PC-OOH was incubated in the presence of Fe^{2+} . Thus, the saliva suppressed Fe^{2+} -induced decomposition of PC-OOH. Changes in the amounts of TBARS in cooked fish by incubating with saline and saliva are shown in Table II. After incubating with saline, the amount of TBARS increased significantly in each homogenate. However, in the case of dark muscle, a tissue containing a high concentration of TBARS, incubating with saliva did not increase the TBARS level.

In the liposomal suspension, lipid peroxidation was initiated by two systems, an azo initiator and Fe^{2+} plus ascorbic acid. The former system has been frequently used to initiate lipid peroxidation, in which a free radical chain reaction was induced by aqueous peroxy radicals from an azo compound. Lipid hydroperoxides accumulate as a result of this chain reaction.¹²⁾ Thus, the level of lipid hydroperoxides is an index of lipid peroxidation. The result that parotid saliva suppressed the accumulation of PC-OOH in this system (Fig. 1A) demonstrates that this extracellular fluid can inhibit peroxidation of the acyl group

Table II. EFFECTS OF INCUBATING WITH NORMAL SALINE AND HUMAN SALIVA ON THE TBAS LEVEL IN COOKED FISH MEAT

Raw mackerel (*Scomber japonicus*) obtained from the local market was broiled in a gas oven for 10 min, divided into white muscle and dark muscle, and then homogenized with normal saline. To 1.0 ml of the homogenate (52 mg of white muscle, 11 mg of dark muscle), 1.0 ml of saline or saliva was added, and the mixture incubated for 2 hr at 37°C.

Sample	TBARS (Malonaldehyde $\mu\text{mol/g}$ of sample)		
	Before incubation	After incubation	
		With saline	With saliva
White muscle	1.3 $\pm$ 0.1	1.5 $\pm$ 0.1*	1.7 $\pm$ 0.1*
Dark muscle	12.0 $\pm$ 0.6	16.0 $\pm$ 0.3*	11.5 $\pm$ 0.7

* Significant difference from the respective muscle before incubating ($p < 0.05$).

of PC. However, parotid saliva was found to contain a very low amount of tocopherols, which seem to be responsible for scavenging chain-propagating lipid peroxyl radicals.^{13,14} It is therefore likely that the inhibition by human saliva is mainly attributable to the ability for quenching aqueous peroxyl radicals. We presume that the lipid peroxidation-initiating hydroperoxy radical ($\cdot\text{OOH}$)¹⁵ is easily generated by protonation of the superoxide anion under the highly acidic conditions of the human stomach. Therefore, scavenging of the aqueous peroxyl radical by parotid saliva seems to be of importance for protecting against lipid peroxidation-mediated free radical damage in ingested foods. The radical-scavenging action of uric acid^{16,17} accounts, at least partly, for this scavenging effect. Other components such as protein thiols may also participate in scavenging such radicals similar to human plasma.¹⁻³

In many cases, the iron ion is responsible for initiating lipid peroxidation.¹⁸ In this system, ascorbic acid prevents Fe^{2+} from being oxidized, and Fe^{2+} may initiate lipid peroxidation by reacting with preformed lipid hydroperoxides. It is probable that the formation of a reactive lipid alkoxyl radical *via* this reaction promotes the radical chain peroxidation.¹⁹ The inhibition by saliva of Fe^{2+} -induced lipid peroxidation is likely to be derived from trapping of the iron ion, scavenging lipid alkoxyl radicals or converting preformed lipid hydroperoxides to stable hydroxy derivatives. We have clarified that blood plasma has an ability to prevent the action of Fe^{2+} on lipid hydroperoxides.²⁰ The result that saliva suppressed the decomposition of PC-OOH (Table I) suggests that human saliva also contains factors preventing the action of Fe^{2+} . It is very likely that uric acid contributes to this prevention,

because it forms a stable coordination complex with the iron ion.²¹ Human saliva contains salivary lactoperoxidase, which produces hypothiocyanite by using peroxide.⁴ However, the ratio of PC-OH to PC-OOH (PC-OH/PC-OOH) after incubating with saliva was no different from that after incubating with saline (Table I). Thus, parotid saliva appears to have little ability for reducing PC-OOH to the stable hydroxy derivatives.

The inhibition by human saliva of Fe^{2+} -induced lipid peroxidation seems to be of importance in ingested foods. The chance that lipid peroxidation-initiation factors such as metal ions and lipid hydroperoxides are in contact with oxidizable unsaturated fatty acids may be increased by mastication and digestion. Our previous work²² has shown that peroxidation-sensitive phospholipid fractions in muscle foods contained a low level of phospholipid hydroperoxides. Heme iron and non-heme iron are well known to affect the oxidative stability of muscle foods by promoting lipid peroxidation.^{23,24} Igene *et al.*²⁵ have pointed out that cooking meat released a significant amount of non-heme iron from bound heme pigment, which accelerated the lipid peroxidation in cooked meat. The dark muscle of fish is, in particular, highly susceptible to lipid peroxidation because of the high concentration of heme and non-heme iron,^{26,27} and the high concentration of peroxidation-sensitive polyunsaturated fatty acids. Obviously, human saliva can suppress the lipid peroxidation of cooked dark muscle of fish and probably other lipid peroxidation-sensitive foods.

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