Effects of Rat Bile-pancreatic Juice on Fe$^{2+}$-Induced Peroxidation of Phospholipids

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The effects of rat bile-pancreatic juice (BPJ) on Fe$^{2+}$-induced oxidation of soybean phosphatidylcholine (PC) was monitored to investigate the influence of this digestive juice on oxidative damage in the gastrointestinal tract. A large volume of BPJ (50% in the suspension of PC, v/v) suppressed the lipid peroxidation, but a smaller volume had the reverse effect. BPJ could decompose free fatty acid hydroperoxides (FFA-OOH) at a lower concentration (~0.2 mM) completely, although its phospholipase activity liberated FFA-OOH from PC hydroperoxides. Sodium deoxylchoolate enhanced the Fe$^{2+}$-induced oxidation of PC in a concentration-dependent manner when PC was suspended in the buffer. Boiled BPJ suppressed Fe$^{2+}$-induced and peroxyl radical initiated oxidation of sodium deoxycholate micelles of soybean PC up to ~50% (v/v). It was strongly suggested that rat BPJ had a biphasic effect on Fe$^{2+}$-induced oxidation of phospholipids depending on the enhancement by bile salts and the inhibition by antioxidant components with radical-scavenging activity and hydroperoxide-decomposing activity.

In general daily foods are susceptible to lipid oxidation and thereby accumulate a number of products including hydroperoxides and their secondary products. Intake of the lipid oxidation products is believed to be harmful to the human body, because they are toxic and probably carcinogenic.1-3 Their deleterious effects on hepatic function4 and immune response5 have been discovered using rodent animals. Lipid oxidation may also occur in the gastrointestinal tract during mastication and digestion because foods often contain prooxidants such as heme or non-heme iron and lipid hydroperoxides. Bull et al.6 have suggested that lipid oxidation products present in foods or produced in the digestive tract are important in the promotinal effect of high dietary fat intake on colon carcinogenesis.

We reported that human salvia has an inhibitory effect on lipid oxidation7-8 and also suggested that gastric juice has a hydroperoxide-decomposing activity.9-10 Stocker et al.11 and Stocker and Ames12 demonstrated that conjugated bilirubin present in bile is an important antioxidant against lipid oxidation. Bile acid has been reported to act as an antioxidant and enhancer depending on the concentration of lipids to be oxidized.12 However, little is yet known about the effect of secretion of bile-pancreatic juice on oxidation of dietary lipids.

The aim of this study was to investigate the effects of digestive juice, secreted into duodenum, on the status of lipid oxidation before intestinal absorption. We prepared bile-pancreatic juice (BPJ) from rats and investigated its effects on Fe$^{2+}$-initiated peroxidation of soybean phosphatidylcholine (PC) because phospholipids are generally responsible for lipid oxidation in oxidation-sensitive muscle foods13,14 and Fe$^{2+}$ is easily available in these foods by releasing from binding protein such as myoglobin during cooking and digestion.15

Materials and Methods

Materials: Soybean PC, arachidonic acid (99%), and soybean lipoygenase (Type I) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium deoxycholate was from Difco Laboratories (Detroit, MI, U.S.A.). 2,2'-Azobis(2-amidinopropane)hydrochloride (AAPH) was purchased from Wako Pure Chemicals (Osaka, Japan). Phosphatidylcholine hydroperoxides (PC-OOH) were prepared from egg yolk PC (Sigma Chemicals Co.) by the procedure described previously.16 For the preparation of BPJ, a male Wistar rat (10 week old) was anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg, Abbott Laboratories, North Chicago, IL, U.S.A.) and the common bile duct in front of the pancreatic duct was cannulated with PE-10 tubing (Becton Dickinson, Parsippany, NJ, U.S.A.). BPJ was drained into a test tube cooled on ice while the rat was under anesthesia, and kept at ~60°C until it was used in the experiment. The solvents for chromatography were of HPLC grade (Nakarai Chemicals, Kyoto, Japan). Distilled water was further purified by the Milli-Q reagent system (Millipore Corporation, Bedford, MA, U.S.A.). The other reagents were of analytical grade.

Oxidation of soybean PC: Soybean PC was dissolved in n-hexane (2.0 mg in 200 μl), evaporated with nitrogen and finally under vacuum. The residue was dispersed in Tris-HCl buffer 100 μl (0.01 M, pH 7.4) and mixed with a Vortex mixer for 30 s followed by ultrasonic irradiation in an Astrason sonicator W-380 (20 kHz, Heat System-Ultrasonics, Inc., N.Y., U.S.A.) for 1 min. A known amount of BPJ was added to the mixture. The volume was adjusted to 200 μl with physiological saline. In the experiment with boiled BPJ, BPJ was boiled for 10 min in a water bath to inactivate its enzymatic activities and sodium deoxycholate was added to the buffer and physiological saline at the concentration of 38 mM for boiled BPJ. The contents were incubated in the dark at 37°C for 3 min. The oxidation was started by the addition of Fe$^{2+}$ and ascorbic acid (20 μl of 1 mM FeSO$_4$ and 10 mM ascorbic acid dissolved in the buffer) or AAPH (20 μl of 200 mM AAPH in the buffer). Final concentration of the incubation mixture were:

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Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane)hydrochloride; BPJ, bile-pancreatic juice; FFA, free fatty acid; FFA-OOH, free fatty acid hydroperoxides; 15-RPTE, 15-hydroperoxyeicos-5,8,11,13-tetraenoic acid; PC, phosphatidylcholine; PC-OOH, phosphatidylcholine hydroperoxides; TBARS, thiobarbituric acid reactive substances.
PC 11.7 mm, FeSO₄ 0.09 mm, ascorbic acid 0.9 mm or AAPH 18 mm. Incubation of the dispersion was done in the dark at 37°C with continuous shaking. Samples were tested by the thiobarbituric acid reactive substances (TBARS) assay at the definite times. Samples were also withdrawn (10 μl) and injected directly into the HPLC column to monitor the formation of PC-OOH.

TBARS assay. A solution of butyl hydroxytoluene (20 μl, 0.22% in ethanol) was added to the reaction mixture (220 μl) to stop the reaction. Phosphoric acid solution (600 μl, 1%) and 0.67% TBA solution (200 μl) was added to the mixture and then left in a boiling water bath for 30 min. After it had cooled, n-butyralcohol (1.0 ml) was added and mixed vigorously. Absorbance at 532 nm and 520 nm of the upper layer were measured after centrifugation at 3500 rpm for 5 min. The amounts of TBARS were calculated by the difference in absorbance between 532 nm and 520 nm and expressed as μM malondialdehyde. Tetrahydroxypropene was used as the standard compound.

HPLC analysis of PC-OOH. PC-OOH were measured by reverse phase HPLC using octane-bonded silica column (TSK gel, Tosoh, Japan) with UV detection (235 nm) as described before except for the use of a guard column (TSK guardgel Octyl 80T, Tosoh, Japan). PC-OOH as standards were prepared from soybean PC as described before.

TLC analysis of the extracts from BPJ. Egg yolk PC (1.2 μmol) or PC-OOH (1.15 μmol) in n-hexane solution was evaporated with a stream of nitrogen and finally under vacuum. The residue was dispersed in 0.1 M Tris–HCl buffer (0.01 M, pH 7.4) by mixing with a Vortex mixer for 30 s and ultrasonic irradiation for 1 min. BPJ was mixed to the dispersion and then incubated at 37°C in the dark for 2 h. The reaction mixture was extracted by chloroform and methanol and concentrated in vacuo. A sample aliquot of the sample was put onto a TLC plate (Merck Silica Gel PF254, 0.25 mm thick) and developed with the solvent of chloroform–methanol–acetic acid–water (35:15:4:2). The plate was stained by exposing to iodine vapor.

Incubation with FFA-OOH. 15-Hydroperoxyeicosatetraenoic acid (15-HPETE) was prepared by enzymatic oxidation of arachidonic acid using soybean lipoygenase according to Funk et al. 18 A known amount of 15-HPETE in 10 μl of methanol were added to 0.1 ml of Tris–HCl buffer (0.01 M, pH 7.4). After the addition of 100 μl of BPJ, the suspension was incubated in the dark for 30 min at 37°C. The mixture was extracted with chloroform and methanol and the extracts were evaporated in vacuo. The residue was redissolved in the eluting solvent of HPLC for the analysis of 15-HPETE.

Results

Effects of BPJ on the oxidation of soybean PC suspension

A liposomal suspension of soybean PC was mixed with BPJ at different concentrations and exposed to Fe²⁺ and ascorbic acid. Soybean PC for this experiment was unpurified and thus contained preformed PC-OOH at the level of 2.7 mol%. Figure 1 shows a typical example of the effects of rat BPJ on the Fe²⁺-induced oxidation of soybean PC suspension. Without BPJ, the level of TBARS reached a maximum after 2 h. BPJ at 5% (v/v) significantly accelerated the rise of TBARS. However, BPJ at 25% (v/v) did not show such acceleration. The increase of TBARS was obviously suppressed throughout the incubation time in the presence of BPJ at 50% (v/v).

Stability of lipid hydroperoxides in BPJ

Figure 2 shows a typical TLC of the lipid extracts from BPJ with or without addition of PC or PC-OOH. It was confirmed that rat BPJ contained PC, lysophosphatidicholine (lysoPC) and free fatty acid (FFA). Total phospholipid content in this BPJ preparation was about ca. 0.3 mm by a spectrophotometric assay. 19 Disappearance of PC after the incubation indicated that PC was hydrolyzed to lysoPC and FFA. FFA-hydroperoxides (FFA-OOH) emerged on the plate when PC-OOH (5.8 mm) was added to the BPJ and then incubated for 2 h. This indicates that PC-OOH was hydrolyzed to FFA-OOH in BPJ. The recovery of PC-OOH at 0.2 mm after incubation with BPJ was 11% by HPLC. 9 Next, 15-HPETE at the concentration from 0.2 to 10 mm was added to 50% (v/v) BPJ solution and then incubated for 30 min to assess the fate of lipid hydroperoxides in this

![Fig. 1. Effects of BPJ on Fe²⁺-Induced Oxidation of Soybean PC Suspension.](image)

![Fig. 2. TLC of the Extract after Incubation of BPJ in the Presence of PC or PC-OOH.](image)

<table>
<thead>
<tr>
<th>15-HPETE (mm)</th>
<th>% recovery</th>
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<tr>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>2.0</td>
<td>15.9 ± 0.4</td>
</tr>
<tr>
<td>10.0</td>
<td>26.2 ± 1.3</td>
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solution. Although some 15-HPETE at 2 and 10 mM remained in the mixture, its smaller concentration (0.2 mM) disappeared completely after the incubation (Table).

Effects of sodium deoxycholate on \( \text{Fe}^{2+} \) induced oxidation of soybean PC suspension

We selected sodium deoxycholate to discover the effect of bile salts on \( \text{Fe}^{2+} \) induced oxidation of PC suspension. Figure 3 shows the relationship between the concentration of sodium deoxycholate and the % of TBARS in control (no sodium deoxycholate). The amount of oxidation was obtained from the amount of TBARS after 3h of incubation. The amount of oxidation was increased by elevating the concentration of sodium deoxycholate with the saturation curve and reached more than 200% at the concentration of 22 mM.

Effects of boiled BPJ on the oxidation of sodium deoxycholate micelles of PC

Soybean PC micelles were prepared with sodium deoxycholate and were subjected to \( \text{Fe}^{2+} \) induced and an azo compound induced oxidation after the addition of boiled BPJ. The concentration of sodium deoxycholate in the micelles was fixed at 38 mM according to the \textit{in vivo} concentration of bile salts in rat intestine.\textsuperscript{21} Total bile salt concentration in the BPJ preparation was ca. 34 mM by a spectrophotometric assay.\textsuperscript{22}

Figure 4 shows the relationship between the concentration of boiled BPJ and the % of amounts of the oxidation in control (no addition of BPJ). The amounts of oxidation were obtained from the amount of TBARS and that of PC-OOH after 1h of incubation. In both oxidation system, BPJ suppressed the accumulation of TBARS and PC-OOH in a concentration-dependent manner from 4.5% (v/v) to 45% (v/v).

Discussion

Lipid peroxidation in foods is complex and proceeds through an initiation reaction and subsequent radical chain reactions. In this study, lipid peroxidation was induced by \( \text{Fe}^{2+} \) plus ascorbic acid. Although the initiation mechanism of this system is not well defined, one electron transfer from \( \text{Fe}^{2+} \) to lipid hydroperoxides yields lipid alkoxyl radicals, which are able to initiate lipid oxidation by abstracting hydrogen from unsaturated lipids.\textsuperscript{23} One electron transfer from \( \text{Fe}^{2+} \) to the preformed PC-OOH is presumably enough for the induction of oxidation in our model system. This reaction seems to be of importance in muscle foods because traces of lipid hydroperoxides inevitably accumulate in the lipid portion.\textsuperscript{24} \( \text{Fe}^{2+} \) deactivation and elimination of lipid hydroperoxides, in addition to scavenging of peroxyl radicals, seem to account for the inhibition of \( \text{Fe}^{2+} \) induced oxidation.

BPJ was found to act as both enhancer and antioxidant, when oxidation of PC suspension was induced by \( \text{Fe}^{2+} \) and ascorbic acid (Fig. 1). Pancreatic lipase is known to hydrolyze triacylglycerol hydroperoxides resulting in the generation of FFA-OOH.\textsuperscript{25} Our results clearly show that BPJ can eliminate phospholipid hydroperoxides by hydrolytic cleavage resulting in FFA-OOH and lysoPC (Fig. 2). Phospholipase activity of BPJ seems to be responsible for the hydrolysis of PC-OOH. However, little recovery of 15-HPETE at the micromolar level (Table) implies that BPJ has an ability to decompose lipid hydroperoxides responsible for initiating \( \text{Fe}^{2+} \) induced lipid peroxidation, as previously pointed out by Stocker et al.\textsuperscript{11} We demonstrated that both human plasma\textsuperscript{19} and parotid saliva\textsuperscript{8} can reduce FFA-OOH to its hydroxy derivative depending on the concentration of glutathione. However, normal phase HPLC analysis of the extract after incubation of 15-HPETE with BPJ gave
no hydroxy derivative even in the presence of glutathione (data not shown). It can be therefore concluded that rat BPJ has little ability to reduce FFA-OOH to its hydroxy derivative.

To understand the influence of bile salt in BPJ, we examined the effects of sodium deoxycholate on Fe^{2+} induced oxidation of PC suspension. Enhancement by bile salts in BPJ may account for the increase of lipid oxidation of PC suspension at the lower volume of BPJ (Fig. 1), because sodium deoxycholate at low concentration worked as enhancer for Fe^{2+} induced oxidation in the mixture of PC suspension (Fig. 3). Delange et al. \cite{12} have also shown that bile salts act as enhancers at the lower concentration to the lipids. Enhancement effect of bile salts is likely to be derived from the micellar formation resulting in the increase of surface area responsible for the initiation reaction. Anionic deoxycholate may also improve the accessibility of cationic Fe^{2+} to the lipid portion in the micellar structure.

The experiment using boiled BPJ and sodium deoxycholate micelles can exclude the effects of phospholipase activity and bile salts. The fact that boiled BPJ inhibited both of AAPH induced oxidation and Fe^{2+} induced oxidation (Fig. 4) demonstrates that BPJ contains antioxidant components acting as free radical scavengers because this azo compound induced a free radical chain reaction by generating aqueous peroxy radicals. \cite{26} The enhancement effect of sodium deoxycholate was saturated at higher concentrations and antioxidant effects of boiled BPJ were elevated with the increase of its concentration in sodium deoxycholate micelles. Thus, antioxidant components seem to be responsible for the inhibitory effect of BPJ at higher volume. Biliary bilirubin in rats are mostly present in conjugated form as glucuronides at the concentration of 0.35 mmole\(^{-}\)L\(^{-}\) and recognized to act as a chain-breaking antioxidant. \cite{10}

Stocker and Ames \cite{11} have implied that conjugated bilirubin and copper ion at physiological concentrations in bile contribute to the decomposition of dietary lipid hydroperoxides in digestive tract. Thus, it is conceivable that conjugated bilirubin is included in antioxidant components in rat BPJ on Fe^{2+} induced phospholipid peroxidation. The fact that lowering of PC-OOH accumulation by boiled BPJ was more significant than that of TBARS accumulation (Fig. 4) may be explained by the participation of hydroperoxide-decomposing activity of BPJ.

In conclusion, rat BPJ exerts an antioxidant effect and an enhancement effect in Fe^{2+} induced peroxidation of PC suspension. Its larger volume inhibits the oxidation through radical scavenging activity and hydroperoxide-decomposing activity, although micellar formation by bile salts may increase the susceptibility of PC to Fe^{2+} induced peroxidation at its lower volume.

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