

Note

Protective Effect of Antioxidative Liposomes Co-encapsulating Astaxanthin and Capsaicin on CCl₄-Induced Liver Injury

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Our previous study reported that co-encapsulation of potent antioxidants astaxanthin (Asx) and capsaicin (Cap) into liposomes brought about synergistically higher antioxidative activity than the calculated additive activity of each single antioxidant encapsulating liposome. Based on the previous computational chemistry analysis, the synergistic effect was revealed to be resulted from intermolecular interactions between Asx, especially 3*R*,3'*R*-form of Asx stereoisomer (Asx-*R*), and Cap, by which changes of electronic states of the polyene moiety of Asx-*R* were induced. Although liposomes co-encapsulating Asx-*R* and Cap (Asx-*R*/Cap-Lipo) at an optimal ratio clearly showed synergistic antioxidative activity *in vitro*, it is unclear whether the effective antioxidative activity derived from intermolecular interaction between Asx-*R* and Cap is also exerted *in vivo*. Therefore, in this study, we investigated therapeutic potential of Asx-*R*/Cap-Lipo as an antioxidant formulation *in vivo*. For this purpose, we employed carbon tetrachloride (CCl₄)-induced acute liver injury rat model, since CCl₄ is known to cause oxidative damage in liver. CCl₄ administration significantly increased the levels of aspartate transaminase (AST) and alanine aminotransferase (ALT). Intravenous combined administration of liposomes encapsulating Asx-*R* (Asx-*R*-Lipo) and liposomes encapsulating Cap (Cap-Lipo) significantly decreased CCl₄-induced increase of AST and ALT levels. Importantly, the treatment with Asx-*R*/Cap-Lipo tended to show higher protective effect on acute liver injury than combined treatment with Asx-*R*-Lipo plus Cap-Lipo. These results suggest that co-encapsulated Asx-*R* and Cap in liposomal membranes could exert more effective antioxidative activities *in vivo*, and that Asx-*R*/Cap-Lipo would be a hopeful antioxidant formulation for treating reactive oxygen species-related diseases.

Key words antioxidant; astaxanthin; capsaicin; liposome; intermolecular interaction; stereochemistry

INTRODUCTION

Oxidative stress, which is caused by excess reactive oxygen species (ROS), is related to pathological progression of a variety of diseases.¹⁾ Antioxidants can scavenge ROS and are considered to be effective in prevention and treatment of ROS-related diseases. We have previously reported that topical administration of liposomal formulation of the carotenoid astaxanthin (Asx, 3,3'-dihydroxy-dihydroxy- β , β -carotene-4,4'-dione), a more potent antioxidant than other representative carotenoids, is effective for prevention of UV-induced skin damage and for the treatment of dry eye.^{2,3)} We also demonstrated that co-encapsulation of Asx and α -tocotrienol (α -T3), one type of vitamin E, into liposomes can exert synergistically higher antioxidative activity compared with the calculated additive activity of each of the single antioxidant encapsulating liposomes *in vitro*.⁴⁾ As to the mechanism, intermolecular interactions *via* hydrogen bonding between the terminal ring of Asx and the chroman ring of α -T3, and *via* partial interaction between those side chains were revealed to be important.⁵⁾ Recently, we found that co-encapsulation of Asx and an antioxidant capsaicin (Cap) can form intermolecular interaction in liposomal membranes similar to α -T3, and show synergistic antioxidative activity.⁶⁾ From the computational chemistry analysis, the heats of in-

termolecular interaction of an Asx stereoisomer 3*R*,3'*R*-form (Asx-*R*) with Cap were revealed to be highest compared with that of other Asx stereoisomers, indicating that Asx-*R* forms more energetically stable intermolecular interaction with Cap in comparison to other stereoisomers. Also, the analysis indicated that the electronic charge of Asx was predicted to be remarkably changed, especially in its polyene moiety, *via* intermolecular interaction, and the changes of electronic state were also confirmed by shift of the peak of the visible absorption of Asx-*R*. Moreover, by evaluating hydroxyl radical scavenging effect *in vitro*, it was also revealed that liposomes co-encapsulating Asx-*R* and Cap (Asx-*R*/Cap-Lipo) show synergistically higher antioxidative effect than combination of liposomes encapsulating each antioxidant. On the other hand, the activity of liposomes co-encapsulating Asx-*S* and Cap was lower than the calculated additive activity of combination of each antioxidant liposome, which reflects the results of prediction by computational chemistry analysis. Based on these results, we suggested that changes in the electronic state of Asx, especially Asx-*R*, *via* intermolecular interaction with Cap should enhance antioxidative activity of Asx.⁶⁾ However, it is unclear whether the effective antioxidative activity derived from intermolecular interaction between Asx-*R* and Cap is also exerted *in vivo*. Thus, evaluation of the effectiveness of the Asx-*R*/Cap-Lipo *in vivo* is required for application as a therapeutic agent for ROS-related diseases.

Carbon tetrachloride (CCl₄) is known to be metabolized

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in endoplasmic reticulum of hepatic cells to highly reactive trichloromethyl radical and trichloromethyl peroxy radical, resulting in induction of oxidative damage in liver.⁷⁾ Thus, CCl₄-induced liver injury model rats are widely employed for evaluation of protective effect of antioxidants *in vivo*.⁸⁾ Therefore, in this study, we investigated therapeutic potential of Asx-*R*/Cap-Lipo as an antioxidant formulation for ROS-related diseases by using CCl₄-induced acute liver injury model rats.

MATERIALS AND METHODS

Materials Capsaicin (Cap), CCl₄, isoflurane, and transaminase CII-Test Wako were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Egg phosphatidylcholine (EPC) was obtained from NOF Corporation (Tokyo, Japan). All other reagents used in the present study were of the highest grade available commercially.

Preparation of Liposomes Encapsulating Antioxidants Liposomes composed of EPC, Asx-*R* (3*R*,3'*R*-form of Asx), and Cap were prepared by the thin-film method. Chloroform solution containing 20 μmol of EPC, 0.2 μmol of Asx-*R*, and 0.4 μmol of Cap were added to eggplant flask and evaporated to form a thin lipid film. The dried lipid film was hydrated with 2 mL of 10 mM Tris-HCl buffer (pH 7.4) at room temperature, after which the liposomal suspension was freeze-thawed for 3 times. Then, the liposomal suspension was extruded through polycarbonate membrane filters with 100-nm pores (Nuclepore, Cambridge, MA, U.S.A.). The diameters of the liposomes were determined with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). In our previous studies, encapsulation efficiency of Asx into liposomes was determined to be almost 100% by spectrophotometer.⁹⁾ Since both Asx and Cap are highly hydrophobic compounds, we assumed that almost all Asx and Cap were encapsulated into hydrophobic region of liposomal membranes, and such liposomes were used in the subsequent experiments.

Animals Eight-week-old male Wistar rats (180–200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University.

Preparation of CCl₄-Induced Liver Injury Model Rat CCl₄-induced liver injury model rats were prepared in accordance with previous reports with some modification.¹⁰⁾ In brief, rats were anesthetized by intraperitoneal administration of chloral hydrate (400 mg/kg), followed by intraperitoneal injection of 10% CCl₄ solution (1 mL/kg body weight) diluted in liquid paraffin to induce hepatic injury. The CCl₄-injected rats were employed as an acute liver injury model described as follows.

Evaluation of Protective Effect of Liposomes Encapsulating Antioxidants To evaluate protective effect of liposomes encapsulating antioxidants on CCl₄-induced liver injury, the CCl₄-injected rats were intravenously injected with liposomes encapsulating Asx-*R* (Asx-*R*-Lipo), liposomes encapsulating Cap (Cap-Lipo), or liposomes co-encapsulating Asx-*R* and Cap (Asx-*R*/Cap-Lipo) 30 min after CCl₄ injection. The injection dosages of the liposomes and each antioxidant were 25 μmol EPC/kg, 0.5 μmol Asx/kg, and 1 μmol Cap/kg, respectively. In the case of the group of combination treatment with Asx-*R*-Lipo and Cap-Lipo, the total amounts of EPC, Asx, and Cap were adjusted to the dosages as mentioned above.

Twenty-four hours after liposomal injection, blood samples were collected from the heart of each rat under isoflurane anesthesia. Three hours after incubation at room temperature, the blood samples were centrifuged at 1000×*g* for 10 min at 4°C, followed by collection of serum. The enzymatic activities of aspartate transaminase (AST) and alanine aminotransferase (ALT) were determined using transaminase CII-Test Wako according to the manufacturer's instructions.

Statistical Analysis Statistical differences were analyzed by one-way ANOVA followed by the Tukey *post-hoc* test. Data were presented as mean ± standard derivation (S.D.).

RESULTS AND DISCUSSION

Asx is known to exhibit three stereoisomers, namely 3*R*,3'*R*-form (Asx-*R*), 3*S*,3'*S*-form (Asx-*S*), and 3*R*,3'*S*-*meso* form (Asx-*meso*).¹¹⁾ We previously reported that the Asx stereochemistry is crucial for intermolecular interaction with Cap in liposomal membranes, and that co-encapsulation of Asx-*R* and Cap at a ratio of 1:2 showed most clear synergistic antioxidative activity.⁶⁾ Based on our previous results, we used Asx-*R*/Cap-Lipo (Asx-*R*/Cap = 1/2M ratio) and each single antioxidant-encapsulating liposome in this study. Since Asx and Cap are both very hydrophobic and cannot be dissolved in water, the antioxidants cannot be administered to the bloodstream in free form. In particular, Asx can be dissolved only in limited organic solvents such as chloroform. Hence, we encapsulated the antioxidants into hydrophobic region of liposomal membranes to evaluate the protective effect against CCl₄-induced liver injury by systemic administration. The structural formulas of Asx, Asx-*R* and Cap are shown in Fig. 1. The particle sizes of each liposome were adjusted to approximately 110–120 nm by extrusion (Table 1). Each liposomal sample were intravenously injected into the rats 30 min after intraperitoneal injection of CCl₄. At 24 h after liposome injection, serum samples were harvested from each rat, followed by determination of the enzymatic activities of AST

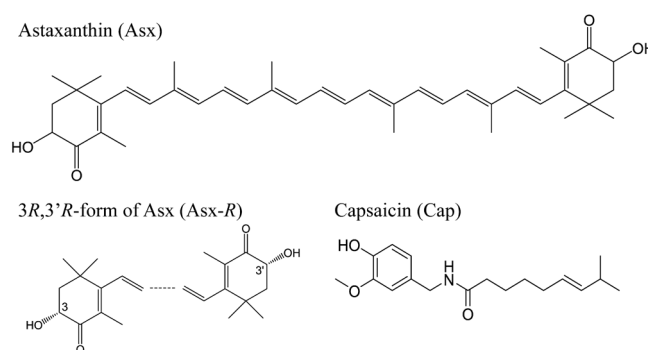


Fig. 1. Structural Formulas of Astaxanthin (Asx), 3*R*,3'*R*-Form of Asx Stereoisomer (Asx-*R*), and Capsaicin (Cap)

Table 1. Particle Sizes of the Antioxidant Liposomes

	Size (d.nm)	Polydispersity index
Asx- <i>R</i> -Lipo	111.2 ± 2.7	0.08 ± 0.01
Cap-Lipo	119.5 ± 5.1	0.08 ± 0.02
Asx- <i>R</i> /Cap-Lipo	113.6 ± 1.3	0.07 ± 0.01

Data are mean ± S.D. (*n* = 4).

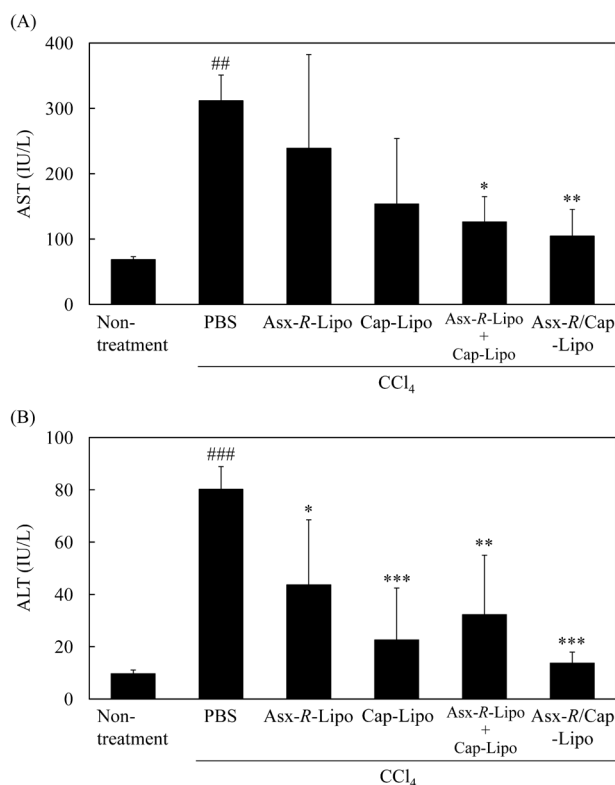


Fig. 2. Effect of Liposomes Co-encapsulating Asx-R and Cap on CCl₄-Induced Liver Injury Model Rat

10% CCl₄ solution (1 mL/kg body weight) was intraperitoneally injected into the rats to induce liver injury. Thirty minutes after CCl₄ injection, the indicated samples were intravenously administered into the rats. The injection dosages of the liposomes and each antioxidant were 25 μmol EPC/kg, 0.5 μmol Asx-R/kg, and 1 μmol Cap/kg, respectively. Twenty-four hours after sample injection, serum samples were collected, followed by determination of the enzymatic activities of AST (A) and ALT (B). Data are mean ± S.D. ($n=4-6$). Significant differences: ^{###} $p < 0.01$, ^{####} $p < 0.001$ vs. Non-treatment; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. PBS.

and ALT to evaluate protective effect of each antioxidative liposome. The results showed that CCl₄ injection significantly increased the values of AST and ALT compared with non-treated rats (Fig. 2), indicated that acute liver injury model rats were successfully prepared. The treatment with Asx-R-Lipo or Cap-Lipo tended to decrease AST level, and significantly decreased ALT level compared with PBS-treated group. Combined administration of Asx-R-Lipo plus Cap-Lipo significantly decreased CCl₄-induced increase of AST and ALT levels. On the other hand, Asx-R/Cap-Lipo treatment tended to show higher protective effect on CCl₄-induced acute liver injury, although the differences were not significant. Based on these results, it is suggested that Asx-R and Cap would efficiently scavenge CCl₄-induced radicals *via* formation of intermolecular interactions in liposomal membranes. Accordingly, the treatment with Asx-R/Cap-Lipo tended to show superior protective effect on CCl₄-induced acute liver injury compared with the groups of each liposome alone and combined treatment. When the antioxidant liposomes were injected into the bloodstream, most of the injected liposomes could be taken up by macrophages, while some of the liposomes could be delivered to hepatocytes and exert the antioxidative activity in liver. Thus, to increase the therapeutic effect of the antioxidant liposomes on liver injury, their modification with hepatocyte targeting ligands such as galactose would be a useful strategy.

Although we examined *in vivo* efficacy of Asx-R/Cap-Lipo in this study, combination of other antioxidants that can form intermolecular interactions in liposomal membranes may allow for preparing more potent antioxidant formulations. This is an interesting issue for future studies. In conclusion, the present study suggests that co-encapsulated Asx-R and Cap in liposomal membranes would have tendency to exert more effective antioxidative activities than combined treatment with Asx-R-Lipo and Cap-Lipo *in vivo*, and that Asx-R/Cap-Lipo would be a promising antioxidant formulation for the treatment of ROS-related diseases.

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Conflict of Interest The authors declare no conflict of interest.

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