Original Article

The anti-apoptotic effect of fucoxanthin on carbon tetrachloride-induced hepatotoxicity

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ABSTRACT — This study evaluated the anti-apoptotic activity of fucoxanthin in carbon tetrachloride (CCl₄)-induced hepatotoxicity. An *in vitro* study using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay clearly demonstrated an attenuation of CCl₄-induced hepatotoxicity with fucoxanthin. This effect was dose-dependent; 25 μM was more effective than 10 μM of fucoxanthin for attenuating the hepatotoxicity induced by 5 mM of CCl₄. Acute CCl₄-hepatotoxicity in rats, with numerous cells positive for the terminal deoxynucleotidyl - transferase (TdT) -mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL) stain were seen in the pericentral area of the hepatic lobule. Oral pretreatment of CCl₄- injected rats with fucoxanthin significantly reduced hepatocyte apoptosis. Fucoxanthin was immunohistochemically shown to increase heme oxygenase-1 expression in the cultured liver cells of Hc cells and TRL1215 cells. By oral pretreatment of CCl₄-injected rats with fucoxanthin, the hepatic heme oxygenase-1 protein levels were significantly increased compared to those not pretreated with fucoxanthin. Heme oxygenase-1 mRNA expression after CCl₄ injection was higher in the CCl₄+fucoxanthin group than in the CCl₄ group, although the difference was not significant. The findings suggest that fucoxanthin attenuates hepatocyte apoptosis through heme oxygenase-1 induction in CCl₄-induced acute liver injury.

Key words: Fucoxanthin, Apoptosis, Heme oxygenase-1, Carbon tetrachloride, Hepatotoxicity

INTRODUCTION

Fucoxanthin is a marine carotenoid found in wakame (*undaria pinnatifida*) and Hijiki (*Hijikia fusiformis*) and has certain remarkable biological properties, including antioxidant activity (Miyashita, 2009; Kim *et al.*, 2010), an anti-inflammatory effect (Sakai *et al.*, 2009), an anti-carcinogenic effect (Hosokawa *et al.*, 2004; Kotake-Nara *et al.*, 2005; Yu *et al.*, 2011) and even utility as an anti-obesity agent (Maeda *et al.*, 2005; Abidov *et al.*, 2010). The major structural difference between fucoxanthin and the other carotenoids is the presence of an unusual allenic bond, and this bond was shown to be responsible for the higher antioxidant activity of fucoxanthin (Dembitsky and Maoka, 2007; Sachindra *et al.*, 2007). The difference, however is that fucoxanthin acts as an antioxidant under hypoxic conditions, whereas other carotenoids have practically no quenching abilities. Under hypoxic conditions, fucoxanthin equimolar-

ly reacted with 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a radical quencher, whereas other carotenoids scarcely reacted with DPPH (Nomura *et al.*, 1997). As most tissues under physiological conditions have a low oxygen presence, fucoxanthin is suggested to have clinical potential as a therapeutic carotenoid for reactive oxygen species (ROS)-associated disorders (D'Orazio *et al.*, 2012; Abidov *et al.*, 2010). It is worth mentioning that fucoxanthin also contains an α, β- unsaturated carbonyl group, and may function as a Michael acceptor which can react with important proteins such as Keap 1 in the nuclear factor E2-related factor-2 (Nrf2) system. A recent *in vitro* study showed that fucoxanthin enhanced heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone 1 expression in murine hepatic BNL CL.2 cells through the activation of the Nrf2/ antioxidant response element (ARE) (Liu *et al.*, 2011).

Carbon tetrachloride (CCl₄) is a well-known chemical that is used for the induction of acute and chronic liver

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injury characterized by pericentral and midzonal necrosis (Slater, 1966). The trichloromethyl radical generated during the metabolism of CCl_4 interacts with oxygen to form the trichloromethyl peroxy radical, a highly reactive species. Trichloromethyl and its peroxy radical are capable of binding to proteins and lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage (Wong *et al.*, 1995; Manibusan *et al.*, 2007). It is well known that oxygen partial pressure can direct the course of CCl_4 hepatotoxicity, and the location of CCl_4 -induced damage mirrors the oxygen gradient across the hepatic lobule (Ozaki and Masuda, 1993). Cellular responses to toxin involve a number of defensive processes aimed at minimizing cell damage and redressing the balance of homeostasis, and the toxicity is commonly manifested in part as cellular apoptosis (Thomas *et al.*, 2001). Hepatocyte apoptosis is likely to play an important role in the development of CCl_4 -induced liver injury and sometimes either precedes the onset of necrosis or coexists with it (Herrera *et al.*, 2001; Weber *et al.*, 2003). Therefore, understanding the mechanism of hepatocyte apoptosis is one of the primary goals related to the designing of future therapies for hepatic injury.

The Keap1/Nrf2/ARE signaling pathway has emerged as an important regulator of the mammalian defense system for the detoxification of agents such as CCl_4 , acetaminophen and furosemide, enabling adaptation to chemical and oxidative stress (Goldring *et al.*, 2004; Lee and Johnson, 2004; Randle *et al.*, 2008; Wen *et al.*, 2006). Under normal physiological conditions, Nrf2 resides in the cell cytoplasm where it associates with a repressor protein, Keap1 (Itoh *et al.*, 1999). Upon disruption of this interaction, Nrf2 is released and migrates to the nucleus where it binds to the ARE present in the promoter regions of many stress-activated genes, including HO-1 and the glutamate- cysteine ligase catalytic subunit (Weber *et al.*, 2003). This indicates that the cell is primed to respond to a chemical insult through rapid up-regulation of Nrf2-driven defensive proteins. As was mentioned above, fucoxanthin may participate in the Nrf2/ARE system and act as a radical quencher under hypoxia, so this xanthophyll is considered to have a potentially protective effect against CCl_4 -induced toxic liver injury. In this study, we investigated the anti-apoptotic effect of fucoxanthin on CCl_4 -induced hepatotoxicity using *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Reagents

Chemicals: CCl_4 and fucoxanthin were purchased from the Wako Pure Chemical Industries, Ltd. (Osaka,

Japan). Solutions of CCl_4 were prepared in olive oil (1:3 v/v) before the experiments. Fucoxanthin was dissolved in a solution of 0.5% sodium carboxymethyl cellulose, 0.5% Tween 80 and ultra pure water to obtain a final concentration of 0.5% (w/v). William's medium E and 10% fetal bovine serum (FBS) were obtained from Sigma Aldrich (St Louis, MO, USA). RIPA lysis buffer was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the other chemicals and solvents were of analytical grade. Antibodies to HO-1 (rabbit polyclonal) and heat shock protein 25 (Hsp25) (rabbit polyclonal) were purchased from Stressgen Bioreagents (Ann Arbor, MI, USA) and Enzo Life Science (Farmingdale, NY, USA), respectively. Anti-rabbit IgG and anti-mouse IgG were from GE Healthcare (Buckinghamshire, UK).

Cell culture and experiments *in vitro*

Cell culture

TRL1215 cell line was originally derived from the liver of a new-born Fisher 344 rat (Nagamine *et al.*, 2008) and kindly donated by Prof. Waalkes (MP) (National Cancer Institute at NIEHS, USA). Hc cells (ACBRI 3716) were initiated from normal human liver tissues (Osawa *et al.*, 2001). These cells were cultured in William's medium E, supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Cellular viability assay (MTT Assay) in TRL1215 cells

TRL1215 cells were plated at 1×10^3 cells per well in five 96-well tissue culture plates and grown in 10% FBS fortified media for 48 hr prior to treatment. Fucoxanthin was added directly to the culture medium of each plate to give a final concentration of 0, 0.5, 1, 10 or 25 μM . After 12 hr incubation, cells were exposed to various concentrations of CCl_4 ; 0, 0.1, 0.5, 1.0, 2.0 or 5 mM. The relative number of viable cells in each well was determined 20 hr after the administration of CCl_4 using a 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Briefly, 5 μl of MTT reagent was added to each well. After 4 hr incubation at 37°C, the media were removed and intracellular formazan products were lysed by the addition of 100 μl of dimethyl sulfoxide. The absorbance was recorded at 550 nm using a plate reader (MPRA4, Toso, Tokyo, Japan).

Immunohistochemistry of HO-1 in cultured liver cells

TRL1215 cells and Hc cells were plated at 1×10^4 cells

per well in 4-well chamber slides and grown in 10% FBS fortified media for 48 hr prior to treatment. Cells were exposed to 0 or 5 μ M of fucoxanthin for 24 hr, and then HO-1 was stained immunohistochemically as follows. The plates were treated with methanol containing 0.3% H₂O₂ for 30 min to eliminate endogenous peroxidase activity. The plates were immersed in hot 0.01 mol/l citrate buffer (pH 6.0) and heated in a microwave for antigen retrieval. Then the plates were incubated with antibodies against HO-1 for 120 min at room temperature. After washing 3 times using PBS with 0.01% Tween 20 (PBS-T), the plates were incubated in a color reaction solution (150 ml of 0.05 mol/Tris-HCl buffer, 30 mg diaminobenzidine, 10 μ l of H₂O₂) for 30 min at room temperature. Finally, the plates were counter-stained with Mayer's hematoxylin.

Animals and treatment procedures

Male Wistar rats (8 weeks of age) were purchased from CLEA Japan. Inc. (Tokyo, Japan). They were housed in the animal facility of Gunma University at a constant temperature and under a 12/12-hr light/dark cycle, and maintained on a standard CE-2 diet purchased from CLEA Japan. Inc. (Tokyo, Japan). The rats were acclimated for 1 week prior to use. All animal procedures were carried out in accordance with the guidelines for experiments using experimental animals at Gunma University.

After an over night fast, rats were randomly assigned to four experimental groups; control (olive oil) group (n = 3), fucoxanthin group (n = 3), CCl₄ group (n = 5) and CCl₄+fucoxanthin group (n = 5). The fucoxanthin and CCl₄+fucoxanthin groups were administered 50 mg/kg (B.W) of fucoxanthin by direct stomach intubation. Four hour later, the CCl₄ group and CCl₄+fucoxanthin group were injected intraperitoneally with 0.25 ml/kg (B.W) of CCl₄ solution. The olive oil group received an equal volume of olive oil intraperitoneally. At 24 hr post-injection, the rats were anesthetized with ether and blood was collected from the jugular vein. The liver was surgically removed and a random sample from each lobe was fixed in 10% formalin, and then embedded in paraffin for hematoxylin-eosin (HE) staining and immunohistochemical staining. The remaining samples were kept frozen at -80°C until the biochemical and gene expression analyses.

Liver function tests along with fucoxanthin and fucoxanthinol levels

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in sera were measured using commercially available kits. The fucoxanthin and fucox-

anthinol levels in sera were analyzed in two rats of the CCl₄+fucoxanthin group and two rats of the olive oil group using a modified method reported previously (Asai *et al.*, 2008; Hashimoto *et al.*, 2009). In brief, 500 μ l of serum was mixed with 1.0 ml methanol and 2.0 ml dichloromethane, and the lower dichloromethane layer was collected. The serum was added 2.0 ml dichloromethane, then the lower layer was collected again. Both collected samples were used for assay. After evaporation, the residue from the dichloromethane layers was dissolved in 200 μ l ethanol. The amounts of fucoxanthin and fucoxanthinol in the ethanol solution were measured by liquid chromatography/ mass/ mass spectroscopy (LC/MS/MS) using a HPLC system (1100 series, Agilent Technologies, Santa Clara, CA, USA) and a QTrap mass spectrometer (API-4000, Applied Biosystems, Foster City, CA, USA). HPLC was performed on a TSKgel ODS-100V column (150 $\times$ 2.0mm i.d., 3 μ m, TOSOH Co. Ltd, Tokyo, Japan) at 30°C with acetonitrile/water (8:2) at a flow rate of 0.4 ml/min. The injection volume was 10 μ l. MS/MS detection was carried out with an atmospheric pressure chemical ionization (APCI) source. The APCI source was set in negative ionization mode. Quantitation was performed using multiple reaction monitoring mode of the transitions of *m/z* 658 $\rightarrow$ 598 for fucoxanthin and *m/z* 616 $\rightarrow$ 558 for fucoxanthinol, respectively.

Liver pathology and immunohistochemistry

After deparaffinization, liver samples were cut into 4- μ m sections and stained with hematoxylin-eosin. Two independent observers evaluated 5 optical fields ($\times$ 200 magnification) in each section chosen randomly under light microscopy, and counted the number of Councilman bodies per field. Then the mean number of Councilman bodies per section was calculated.

For immunohistochemistry, the sections were pre-treated for 20 min with 3% hydrogen peroxide in PBS to quench any endogenous peroxidase activity. Then the sections were subjected to immunohistochemical staining using antibodies against HO-1 or Hsp 25, as mentioned above.

Areas of positive staining were quantified with ImageJ 1.38 x software (Wayne Rasbans, NIH, Bethesda, USA). The ratios of the positively stained areas were calculated from the ratio of the positive staining (brown color by immunohistochemistry) to the whole area of the liver captured in digital images. Three ocular fields ($\times$ 400 magnification) per section were selected randomly from all of the samples, and the mean percentage of HO-1 positive cells per section was calculated. For the analysis of Hsp 25 positive cells, three ocular fields ($\times$ 200 magnifi-

cation) per section were selected randomly, and the mean percentage of Hsp 25-positive cells was calculated.

TUNEL staining

Apoptotic bodies in the liver were detected by terminal deoxynucleotidyl - transferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL), which was performed using an In situ apoptosis detection kit (Roche Diagnostics). For analysis of the TUNEL- positive ratio, two independent observers evaluated 5 optical fields ($\times 400$ magnification) for each specimen chosen randomly under light microscopy. Then the mean number of TUNEL-positive cells per specimen was calculated.

Metallothionein 1/2 values in liver tissues

Metallothionein (MT)1/2 values in liver tissues were assayed using the competitive ELISA according to the method described previously (Nakajima *et al.*, 2010). Briefly, 50 μ l of sample specimen and 50 μ l of MT antibody were added per well and incubated for 1 hr at 4°C in a microplate coated with rabbit MT2A. After incubation, wells were washed 3 times with PBS-T. Then, 100 μ l of HRP conjugated anti-rabbit IgG goat antibody was added per well and incubated for 1 hr at room temperature. After incubation, wells were washed with PBS-T, and 100 μ l of color substrate 3,3',5,5'-tetramethylbenzine were added and reacted for 10 min at room temperature. After the reaction, 50 μ l of 1N H₂SO₄ was added to stop the reaction. Optical density was read at a 450 nm wave-length by a plate reader (MPRA4, Toso, Tokyo, Japan)

Western blot analysis

Liver tissues were homogenized in 3X volume of RIPA lysis buffer and centrifuged at $10,000 \times g$ for 15 min at 4°C, and the supernatant was recovered for the Western blotting of HO-1 and β -actin. Proteins were separated using 12.5% SDS PAGE and transferred to PVDF membranes. Then the blotted membranes were blocked in Amersham ECL Prime Blocking Agent at 2% in T-PBS overnight at 4°C. After washing 3 times with PBS-T, the membranes were incubated for 1 hr at room temperature with an anti-HO-1 rabbit antibody (1:10,000) or anti- β -actin mouse antibody (1:10,000). Subsequently, the membrane was washed with PBS-T and was stained for 1 hr at room temperature using peroxidase labeled anti-rabbit IgG (1:150,000) or anti-mouse IgG (1:75,000). After washing with PBS-T, the bound antibodies were visualized with the ECL-Prime Western Blotting Detection Reagent according to the manufacturer's protocol (GE Healthcare, Buckinghamshire, UK). Densitometry was

used to measure the relative optical density of each specific band obtained after Western blotting.

Real time polymerase chain reaction (real time-PCR)

Quantitative real time-PCR was conducted in 10 μ l reactions containing LightCycler 480 SYBR Green I Master (Roshe Applied Science, Indianapolis, IN, USA) using the LightCycler 480 (Roshe Applied Science, Indianapolis, IN, USA).

The oligonucleotide primers for HO-1, hypoxia inducible factor(HIF)-1 α and GAPDH were as follows:

HO-1 Forward, 5'- CTGTGTAACCTCTGCTGTTCC -3'
HO-1 Reverse, 5'- CCACACTACCTGAGTCTACC -3'
HIF-1 α Forward, 5'-TGCTCATCAGTTGCCACTT-3'
HIF-1 α Reverse, 5'-TGGGCCATTCTGTGTGTA-3'
GAPDH Forward 5'- ATGGTGAAGGTCGGTGTGAAC
GAPDH Reverse 5'-TGTAGTTGAGGTCAATGAAGG-3'

Reaction mixtures were incubated for an initial denaturation at 95°C for 10 sec, followed by 40 PCR cycles. Each cycle consisted of 95°C for 10 sec and 60°C for 10 sec, and 72°C for 10 sec, then 72°C for 10 min. The expression level of each mRNA was normalized by comparison with that of GAPDH. And samples were quantified using standard cDNA dilutions.

Statistical analysis

Data were analyzed with Dr SPSS II (SPSS, Chicago, IL, USA). The statistical significance of difference was determined with the Tukey-Kramer test and Games-Howell test after 1-way ANOVA. Probabilities of $p < 0.05$ were regarded as statistically significant.

RESULTS

MTT assay

Proliferation of TRL1215 cells was inhibited by CCl₄ in a dose- dependent manner (Fig. 1). Pretreatment with more than 10 μ M fucoxanthin attenuated the growth inhibition of TRL1215 cells by CCl₄, while 0.5 and 1.0 μ M of fucoxanthin did not ameliorate CCl₄-hepatotoxicity. In the case of hepatotoxicity induced by 2 or 5 mM of CCl₄, a high concentration of fucoxanthin (25 μ M) markedly reduced the hepatotoxicity compared to 10 μ M of fucoxanthin.

Immunohistochemistry in Hc cells and TRL1215 cells

HO-1 was clearly stained within the cytoplasm of Hc cells, and HO-1 expression was increased 24 hr after the administration of 5 μ M fucoxanthin. In TRL1215 cell, the immunoreactivity of the HO-1 antibody was weak, and

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Fig. 1. Effect of fucoxanthin on CCl₄-induced hepatocyte toxicity (MTT assay). The proliferation of TRL1215 cells was inhibited by CCl₄ in a dose-dependent manner. Pretreatment with more than 10 μM of fucoxanthin attenuated the growth inhibition of TRL1215 cells by CCl₄. At 5 mM of CCl₄-hepatotoxicity, 25 μM of fucoxanthin markedly reduced CCl₄-hepatotoxicity compared to 10 μM of fucoxanthin.

the intensity of the HO-1 stain was slightly increased by the administration of 5 μM fucoxanthin (Fig. 2).

Liver function tests, and the fucoxanthin and fucoxanthinol levels in rats

The serum ALT levels (UI/l) in rats were significantly ($p < 0.05$) elevated in the CCl₄+fucoxanthin group (211.8 ± 67.4) and the CCl₄ group (247.8 ± 47.6) compared to the olive oil group (40.0 ± 7.0). The serum AST levels (UI/l) were also significantly elevated in the CCl₄+fucoxanthin group (444.0 ± 110.2) and the CCl₄ group (482.6 ± 21.7) compared to the olive oil group (91.3 ± 3.5). The CCl₄+fucoxanthin group displayed lower ALT and AST levels than the CCl₄ group, although the difference was not significant. Fucoxanthin alone did not alter the ALT (37.3 ± 4.2) or AST levels (151.0 ± 61.4).

Fucoxanthin and its metabolite fucoxanthinol were detected in the sera of rats 28 hr after the oral administration of 10 mg fucoxanthin. The fucoxanthin levels (0.322 and 0.330 μg/ml) were higher than the fucoxanthinol levels (0.112 and 0.180 μg/ml). Neither fucoxanthin nor fucoxanthinol was detected in the sera of the olive oil-treated rats.

Liver pathology

CCl₄-injected rat livers exhibited degenerative and/or necrotic hepatocytes concomitant with inflammatory cell infiltration and steatosis in the pericentral area. Hydrophic degeneration of the hepatocytes was observed around the apoptotic and/or necrotic hepatocytes. The lesions were similar in the CCl₄ and CCl₄+fucoxanthin groups. Council-

Fig. 2. Immunohistochemistry of HO-1 in liver cell cultures ($\times 200$). Reactivity for the HO-1 antibody was evident in Hc cells and weak in TRL1215 cells. HO-1 expression was mildly increased by 5 μM of fucoxanthin. a, Hc cells + 0 μM of fucoxanthin. b, TRL1215 cells + 0 μM of fucoxanthin. c, Hc cells + 5 μM of fucoxanthin. d, TRL1215 cells + 5 μM of fucoxanthin.

man bodies, regarded as a sign of the apoptotic process (Kerr *et al.*, 1979; Kanzler and Gall, 2000), appeared sporadically around the central vein in the CCl₄-injected liver (Fig. 3 top left). The number of Councilman bodies was significantly ($p < 0.05$) reduced in the CCl₄+fucoxanthin group compared with the CCl₄ group. No histological changes were found in the liver of the fucoxanthin group or the olive oil group (Fig. 3 top right).

TUNEL staining

The TUNEL-positive nuclei of hepatocytes regarded as apoptotic cells were markedly increased in the pericentral area of the CCl₄ group, whereas TUNEL-positive cells were scarcely seen in the CCl₄+fucoxanthin, fucoxanthin or olive oil group, respectively. The areas of the TUNEL-positive hepatocytes corresponded to the areas where Councilman bodies and/or degenerative hepatocytes were observed (Fig. 3 bottom left). The number of the TUNEL-positive hepatocyte nuclei in the CCl₄ group was significantly ($p < 0.01$) higher than in the CCl₄+fucoxanthin group. However, there was no difference in the number of TUNEL-positive cells among the CCl₄+fucoxanthin, fucoxanthin and olive oil groups (Fig. 3 bottom right).

Immunohistochemistry of HO-1 in rat liver

HO-1 was stained mainly in Kupffer cells and sporadically in the nuclei of hepatocytes, and the number and

intensity of the HO-1-positive cells were increased after CCl₄ injection. In the CCl₄+ fucoxanthin group, numerous Kupffer cells strongly reacted to HO-1, but HO-1-positive cells were rarely observed in the fucoxanthin group or the olive oil group (Fig. 4 top left). The percentage of HO-1-positive cells was significantly higher in the CCl₄+ fucoxanthin group than the CCl₄ group ($p < 0.05$), the fucoxanthin group ($p < 0.01$) and the olive oil group ($p < 0.05$). There was no significant difference in the percentage of HO-1-positive cells among the CCl₄, fucoxanthin and olive oil groups (Fig. 4 top right).

Western blotting of HO-1 in rat liver

The HO-1 protein was significantly ($p < 0.05$) increased in the CCl₄+ fucoxanthin group compared to the CCl₄ group, was similar between the CCl₄ group and the control (Fig. 4 bottom).

Immunohistochemistry of Hsp25 in rat liver

The Hsp25 protein was stained in both the cytoplasm and nuclei of the hepatocytes in the pericentral area of the CCl₄ injected liver, an effect which was evidently reduced in rats with pretreatment of fucoxanthin compared to those without fucoxanthin. There was no staining of Hsp25 in hepatocytes of the fucoxanthin group or the olive oil group (Fig. 5 left). The percentage of Hsp25-positive cells was significantly ($p < 0.05$) higher in the CCl₄ group than the CCl₄+fucoxanthin group (Fig. 5 right).

MT values in liver tissues

MT1/2 values in liver tissues were unchanged by CCl₄ injection, and, administration of fucoxanthin did not alter MT1/2 values in rat liver [MT1/2 values ($\mu\text{g/g}$): the CCl₄+ fucoxanthin group 27.1 ± 8.9 , the CCl₄ group 25.7 ± 7.8 , the fucoxanthin group 22.4 ± 3.1 , the olive oil group 35.4 ± 10.1].

Real time RT-PCR

HO-1 mRNA was slightly increased 24 hr after CCl₄ injection. HO-1 mRNA expression was higher in the CCl₄+fucoxanthin group than the CCl₄ group, although the difference was not significant. Hypoxia inducible factor (HIF)-1 α mRNA expression was unchanged by CCl₄ with/without fucoxanthin (Fig. 6).

DISCUSSION

Since CCl₄-induced liver injury is a commonly used model for the screening of the antihepatotoxic/hepatoprotective activities of drugs (Goldring *et al.*, 2004; Lee and Johnson, 2004; Wen *et al.*, 2006; Randle *et al.*,

2008), we examined the effect of fucoxanthin on CCl₄-induced hepatotoxicity. In the present study, MTT assay clearly demonstrated that fucoxanthin attenuated CCl₄-induced hepatotoxicity. This effect was dose-dependent; 25 μM of fucoxanthin was more effective than 10 μM for the hepatotoxicity induced by 2 or 5 mM of CCl₄. On the other hand, low concentrations, such as 0.5 and 1.0 μM of fucoxanthin, did not attenuate CCl₄-hepatotoxicity. In CCl₄-injected rats, Councilman bodies, known as sign of the apoptotic process (Kerr *et al.*, 1979; Kanzler and Gall, 2000), and apoptotic hepatocytes positive for TUNEL staining, were markedly evident in the centrilobular zone 3 area of the liver. No councilman bodies and few hepatocytes undergoing apoptosis were found in the liver specimens of the fucoxanthin and olive oil groups. This result confirmed the findings of previous studies (Irie *et al.*, 2010; Srilaxmi *et al.*, 2010), in which apoptotic hepatocytes were seen in zone 3 after the injection of a single dose of CCl₄. The present study clearly showed that pretreatment of CCl₄-injected rats with fucoxanthin significantly ($p < 0.05$) reduced the presence of Councilman bodies and hepatocyte apoptosis. Hepatocyte apoptosis in the CCl₄+fucoxanthin group was attenuated to an extent close to the olive oil group. In other words, CCl₄-induced hepatocyte apoptosis was apparently prevented by pretreatment with fucoxanthin, suggesting that fucoxanthin has an anti-apoptotic effect and inhibited the toxic damage. There have been a number of reports on the anti-cancer properties of fucoxanthin through apoptosis induction (Hosokawa *et al.*, 2004; Kotake-Nara *et al.*, 2005; Yu *et al.*, 2011), while the anti-apoptotic effect of fucoxanthin was reported in only a single *in vitro* study (Heo and Jeon, 2009). This is the first study to demonstrate the anti-apoptotic effect of fucoxanthin *in vivo*.

The anti-apoptotic mechanism of the effect of fucoxanthin on CCl₄-induced acute hepatotoxicity remains to be elucidated; however, HO-1 induction by fucoxanthin has attracted interest in terms of protection against CCl₄-induced hepatotoxicity. The HO-1 induced by CCl₄ degrades heme to carbon monoxide, which possesses anti-inflammatory, anti-proliferative and anti-apoptotic activities (Farombi and Surh, 2006). Indeed, overexpression of HO-1 with glycyrrhizin alleviates CCl₄-induced acute liver injury in mice (Lee *et al.*, 2007). The present *in vitro* study revealed that 5 μM of fucoxanthin increased HO-1 expression in both Hc cells and TRL1215 cells. In the rat liver 24 hr after CCl₄ injection, HO-1 was markedly expressed, principally in Kupffer cells and sporadically in the nuclei of hepatocytes. The ratio of HO-1-positive cells significantly ($p < 0.05$) higher in rats treated with CCl₄ and fucoxanthin than with CCl₄ alone. In addition,

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Fig. 3. HE staining ($\times 400$) and TUNEL staining ($\times 200$) of rat liver. Top: a representative photomicrograph of the liver histology stained with HE in each group (a. CCl₄+fucoxanthin group, b. CCl₄ group, c. fucoxanthin group, d. olive oil group). Necrosis and steatosis of the hepatocytes and inflammatory cell infiltration are seen in the pericentral area of the lobule in the CCl₄-injected rat. Hydrophic degeneration of hepatocytes is scattered around the area of hepatocyte necrosis. Councilman bodies appear sporadically around the central vein in the CCl₄-injected liver. No changes are evident in the liver of the fucoxanthin group or olive oil group. The arrow indicates the Councilman bodies (left). The number of Councilman bodies was significantly decreased in the CCl₄+fucoxanthin group compared to the CCl₄ group (right). Each value (mean $\pm$ S.D., $n = 3-5$) is the number of Councilman bodies per section. Bottom: a representative photomicrographs of TUNEL staining in each group. TUNEL is stained in the nuclei of hepatocytes and is markedly increased in the pericentral area of the CCl₄ group. TUNEL positive cells are only rarely evident in the CCl₄+fucoxanthin group, the fucoxanthin group and the olive oil group (left). The number of TUNEL-positive cells is significantly higher in the CCl₄ group than in the CCl₄+ fucoxanthin group (right). Each value (mean $\pm$ S.D., $n = 3-5$) is the percent of TUNEL-positive cells per section. CCl₄: the CCl₄ group, CCl₄+Fx: the CCl₄+fucoxanthin group, Fx: the fucoxanthin group, olive oil: the olive oil group. * $p < 0.05$ compared with the CCl₄+fucoxanthin group. ** $p < 0.01$ compared with the CCl₄+fucoxanthin group. ## $p < 0.01$ compared with the CCl₄ group.

Fig. 4. HO-1 protein in rat liver. Top: a representative immunohistochemical staining of HO-1 in rat liver (× 200) in each group (a. the CCl₄+fucoxanthin group, b. the CCl₄ group, c. the fucoxanthin group and d. the olive oil group). HO-1 is stained markedly in Kupffer cells and sporadically in the nuclei of hepatocytes in the CCl₄+fucoxanthin group. HO-1-positive cells are rarely observed in the CCl₄, fucoxanthin and olive oil groups (left). The percentage of HO-1-positive cells in the CCl₄+fucoxanthin group was significantly increased compared to those of the other three groups (right). Each value (mean ± S.D., n = 3-5) is the percent of HO-1 positive cells per section. *p < 0.05 compared with the CCl₄+fucoxanthin group. **p < 0.01 compared with the CCl₄+fucoxanthin group. Bottom: Western blotting of HO-1 in rat liver. Equal loading was assessed with an anti-β-actin antibody. The 32 kDa HO-1 band and 49 kDa β-actin band are indicated by arrows. Lane 1-5 the CCl₄+fucoxanthin group. Lane 6-10 the CCl₄ group. Lane 11,12 the fucoxanthin group. Lane 13,14 the olive oil group. Western blots were scanned by densitometry and the data is presented as relative intensity units. Each bar represents the mean ± S.D. of four to five rats. The control consists of two fucoxanthin groups and two olive oil groups. *P < 0.05 compared with the CCl₄+fucoxanthin group.

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Fig. 5. Immunohistochemistry of Hsp25 in rat liver. A representative photomicrograph of Hsp25 staining ($\times 200$) in each group (a. the CCl₄+fucoxanthin group, b. the CCl₄ group, c. the fucoxanthin group and d. the olive oil group) is shown in Fig. 5 left. The Hsp25 protein is stained in the cytoplasm and nuclei of hepatocytes in the pericentral area of the CCl₄ injected liver, which effect is significantly reduced in the rats receiving the pretreatment with fucoxanthin. Hsp25 is not stained in the hepatocytes of the fucoxanthin group or olive oil group. The percentage of Hsp25-positive cells in the CCl₄+ fucoxanthin group is significantly decreased compared to the CCl₄ group (Fig. 5 right). Each value (mean $\pm$ S.D., $n = 3-5$) is the percent of Hsp 25-positive cells per section. * $p < 0.05$ compared with the CCl₄+fucoxanthin group. ** $p < 0.01$ compared with the CCl₄+fucoxanthin group. ## $p < 0.01$ compared with the CCl₄ group.

Fig. 6. HO-1 and HIF-1 α mRNA expression in rat liver. HO-1 mRNA expression in the liver was measured by real time PCR analysis, and samples were quantified using standard cDNA dilutions. HO-1 mRNA expression is higher in the CCl₄+ fucoxanthin group than the CCl₄ group, the fucoxanthin group and the olive oil group, although the difference is not significant. HIF-1 α mRNA expression was unchanged by CCl₄ with/ or without fucoxanthin. The results are presented as the mean $\pm$ S.D.

pretreatment with fucoxanthin significantly ($p < 0.05$) increased the hepatic HO-1 protein content in CCl₄-injected rats. HO-1 mRNA expression was up-regulated in the following order, the CCl₄+fucoxanthin group, the CCl₄ group, the olive oil group and the fucoxanthin group, although the difference between the groups was not significant. This result is similar to one reported by Liu *et al.* (2011), in which fucoxanthin significantly increased HO-1 protein expression, but mildly up-regulated HO-1 mRNA in murine hepatic BNL CL.2 cells after incubation for 24 hr. Sass *et al.* (2003) showed that up-regulation of endogenous HO-1 rescued mice from the apoptotic liver damage induced by anti-CD3Ab, lipopolysaccharide or tumor necrosis factor- α . When these data are taken into consideration together with our *in vivo* and *in vitro* findings, it is suggested that fucoxanthin exerts an anti-apoptotic effect on CCl₄-induced apoptotic hepatocytes via HO-1 induction.

The Keap1-Nrf2-ARE signaling pathway has emerged as an important regulator in the mammalian detoxication of CCl₄ through the up-regulation of the cellular antioxidant enzyme HO-1 (Randle *et al.*, 2008). HO-1 induction

by fucoxanthin is mediated through several pathways. HO-1 induction by fucoxanthin was previously evaluated in detail using liver BNL CL.2 cells (Liu *et al.*, 2011), with the result that fucoxanthin significantly increased the phosphorylation of ERK and p38 and markedly increased nuclear Nrf2 protein accumulation. Moreover, fucoxanthin was shown to significantly enhance the binding activities of nuclear Nrf2 with ARE, and to increase the mRNA and protein expression of HO-1. Furthermore, siRNA inhibition of Nrf2 led to markedly decreased HO-1 protein expression. However, there has been no reported study *in vivo* on HO-1 induction by fucoxanthin. An *in vivo* study is necessary to elucidate whether the oral administration of fucoxanthin is absorbed via the intestinal tract. Sugawara *et al.* (2002) showed that dietary fucoxanthin was deacetylated into fucoxanthinol in the intestinal tract and incorporated as fucoxanthinol into the blood circulation system. A similar metabolic pathway of fucoxanthin was also reported in rats (Sangeetha *et al.*, 2010). The present study detected fucoxanthin and its metabolite of fucoxanthinol in the sera of rats collected 28 hr after oral administration of fucoxanthin, indicating that fucoxanthin, at least partly, was able to be absorbed through the intestinal tract in rats. Since fucoxanthin enhances HO-1 induction in the rat liver, fucoxanthin may exert its antioxidant activity through an activation of the Nrf2/ARE system, as was reported in the cultured hepatocytes (Liu *et al.*, 2011).

Hsp function as molecular chaperones in regulating cellular homeostasis and promoting cell survival (Ellis, 2007). Many studies have demonstrated that Hsp-induced cytoprotection can be attributed in part to the suppression of apoptosis (Concannon *et al.*, 2001). Hsp25 is one of the major classes of Hsp families in the rat and mouse, a homolog for human Hsp27, and the inhibitory effect of Hsp25 on the apoptosis pathway is well known (Salminen *et al.*, 1997; Sreedhar *et al.*, 2004). More recently, the induction of the Hsp25 protein and apoptotic cells were reported in the pericentral area of rat liver after CCl₄ injection (Fujisawa *et al.*, 2012). In line with this report, the present study showed that apoptotic cells, demonstrable by the TUNEL method, were obviously seen in the pericentral area, corresponding to the area where Hsp25 was expressed. However, the marked induction of Hsp25 by CCl₄ in rat liver was significantly ($p < 0.05$) decreased by pretreatment with fucoxanthin, and fucoxanthin alone did not induce Hsp25 expression immunohistochemically. Thus, the anti-apoptotic effect of fucoxanthin is unlikely to be due to Hsp25 induction. Since fucoxanthin acts as an antioxidant under hypoxic conditions (Nomura *et al.*, 1997), we have also investigated hypoxia inducible fac-

tor (HIF)-1 α and metallothionein (MT)1/2 expression, critical hypoxia-induced proteins (Murphy *et al.*, 2008; Adams *et al.*, 2009), in the present study. Consequently, it was also shown that HIF-1 α and MT1/2 did not participate in the anti-apoptotic effect of fucoxanthin on CCl₄-hepatotoxicity.

Hepatocyte apoptosis sometimes precedes the onset of necrosis, or coexists with it, therefore a drug which affords protection against hepatocyte apoptosis also protects against the secondary necrosis of liver tissue (Herrera *et al.*, 2001; Weber *et al.*, 2003). In the present study, the serum AST and ALT levels were lower in the CCl₄+fucoxanthin group than the CCl₄ group, but the difference was not significant, despite a marked suppression for hepatocytes apoptosis. The reason is speculated to be that fucoxanthin markedly suppresses apoptosis but mildly attenuates necrosis in hepatocytes subjected to CCl₄ toxicity.

In conclusion, fucoxanthin, via the induction of HO-1, decisively reduces hepatocyte apoptosis induced by CCl₄. Clarification of the anti-apoptotic pathway mediated by fucoxanthin will require future study. There is increasing evidence that HO-1 is protective in several disparate models of hepatic stress. As the induction of HO-1 expression by fucoxanthin if of potential therapeutic value, further experiments using well designed *in vivo* models to properly evaluate the efficacy of fucoxanthin are warranted.

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