Inflammation, Vol. 37, No. 2, April 2014 (© 2013)
DOI: 10.1007/s10753-013-9757-1

First Evidence for the Anti-inflammatory Activity of Fucoxanthin in High-Fat-Diet-Induced Obesity in Mice and the Antioxidant Functions in PC12 Cells

Cong-ping Tan[1,2] and Yun-hua Hou[1]

Abstract—Obesity, characterized as a state of low-level inflammation, is a powerful determinant influencing the development of insulin resistance and progression to type 2 diabetes. The purpose of the present study was to investigate the anti-inflammatory activity of fucoxanthin in experimental high-fat-diet-induced obesity in mice and antioxidant activity in PC12 cells under oxidative stress situation. The anti-inflammatory potential of fucoxanthin in the regulation of maleic dialdehyde (MDA), polymorphonuclear cells (PMNs), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), and cyclooxygenase-2 (COX-2) was determined by ELISA. Fucoxanthin significantly inhibited obesity-induced upregulation of the production of IL-1β, TNF-α, iNOS, and COX-2. Moreover, fucoxanthin suppressed MDA and infiltration of PMNs. The protective effects were associated with lack of hypertrophy and crown-like structures in mammary gland. At the same time, fucoxanthin showed an advantage of antioxidant activity in PC12 cells under oxidative stress situation. These results suggest that supplementation of fucoxanthin is a promising strategy for blocking macrophage-mediated inflammation and inflammation-induced obesity and its associated complications.

KEY WORDS: fucoxanthin; inflammation; obesity; ELISA.

INTRODUCTION

Work over the past decade has increasingly linked obesity and inflammation. Contrary to an acute response to an injury, which would typically induce pain and swelling, obesity-associated inflammation is a chronic, unmitigated inflammation with insidious results [1–4]. However, it is increasingly being understood that obesity-induced chronic inflammation plays an important role in the development of obesity-induced insulin resistance and type 2 diabetes [5]. It is desirable to find a product that can help patients with obesity-related inflammation and metabolic dysregulation.

Fucoxanthin (Fig. 1) is one of the most abundant carotenoids and contributes more than 10 % of the estimated total production of carotenoids in nature, especially in the marine environment [6]. Earlier studies had indicated that fucoxanthin was an effective radical scavenger [7]. It was suggested that fucoxanthin was an active component for the antiobesity effect [8]. It was also found that fucoxanthin significantly reduced plasma and hepatic triglyceride concentrations and the activities of adipocyte fatty acid synthesis, hepatic fatty acid and triglyceride synthesis, and cholesterol-regulating enzymes [9, 10].

Maeda *et al.* [11] found that fucoxanthin markedly decreased the blood glucose and plasma insulin levels, as well as water intake in diabetic/obese KK-Ay mice. These observations raise another interesting question as to whether fucoxanthin has the protective effects against obesity-induced inflammation. In the present study, we assessed the activity of fucoxanthin to decrease obesity-induced inflammation in mice and the antioxidant activity in PC12 cells under oxidative stress situation.

[1] School of Food Science and Bioengineering, Qilu University of Technology, Jinan 250353, People's Republic of China
[2] To whom correspondence should be addressed at School of Food Science and Bioengineering, Qilu University of Technology, Jinan 250353, People's Republic of China. E-mail: congpingtan@yeah.net

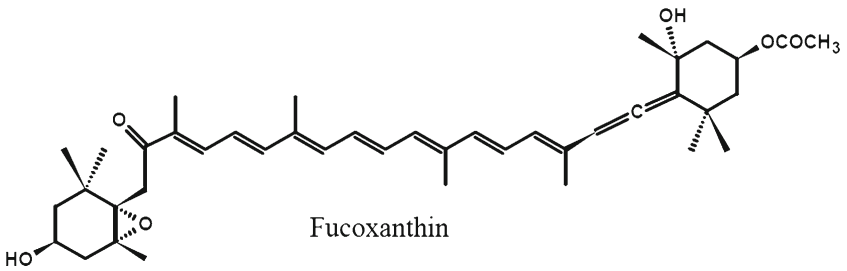

Fig. 1. Structure of fucoxanthin.

MATERIALS AND METHODS

Chemicals

Fucoxanthin was purchased from Leili Natural Products Co., Ltd., China.

Animal

Kunming strain mice weighing 20–22 g were maintained at room temperature under alternating natural light/dark photoperiod and had access to standard laboratory food and fresh water *ad libitum*.

Mice Treatment Protocol

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals. The mice (n=60) were randomly assigned to either continue receiving regular chow (20 % calories from fat, 60 % from carbohydrates, and 20 % from proteins) (C group, n=12) or were fed a high-fat diet (HFD; 60 % calories from fat, 20 % from carbohydrates, and 20 % from proteins) (H group, n=48) for 9 weeks. Thirty-six of the mice consuming the H diet were randomly assigned to receive (intragastric, ig) fucoxanthin (0.2, 0.4, and 0.6 %) (F-0.2, F-0.4, and F-0.6 groups, respectively) for 4 weeks. The other 12 mice were administered (ig) saline (H group, n=12). The body weights of the mice were measured on the zeroth, second, third, and fourth week. Five weeks later, the mice were sacrificed and the blood was collected and serum was stored immediately at −20 °C to estimate inflammatory cells and inflammatory mediators. The mammary glands were formalin-fixed for histological analyses.

Light Microscopy

Four micron-thick sections were prepared from formalin-fixed, paraffin-embedded mammary gland tissue and stained with hematoxylin and eosin. The total number of crown-like structures (CLS) per section was quantified by a pathologist (DG), and the amount of adipose tissue present on each slide was determined using NIH ImageJ. Inflammation was quantified as CLS per square centimeter of adipose tissue.

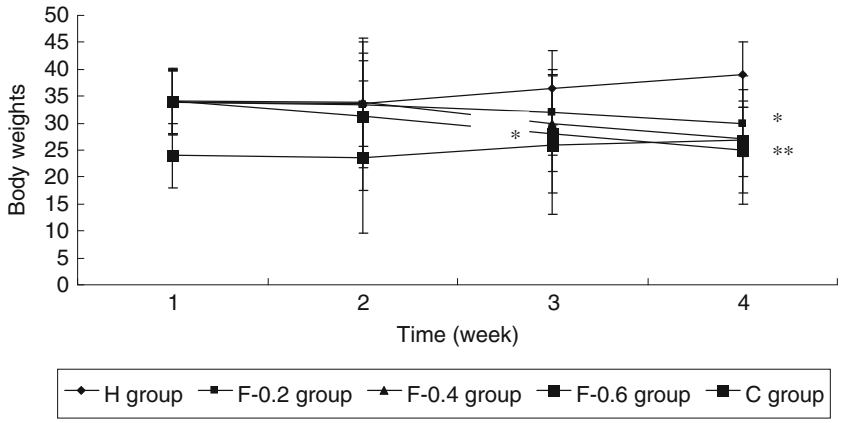

Fig. 2. Effects of fucoxanthin on body weights of mice. Values are shown as means ± SEM. *P<0.05 *vs.* H group, **P<0.01 *vs.* H group.

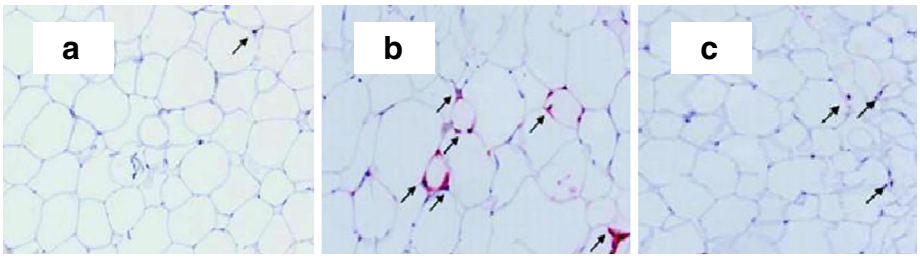

Fig. 3. Mammary gland inflammation is reversed by caloric restriction (*arrowheads* point to crown-like structures). **a** Representative pictures of H&E staining for adipose tissues of the C group. **b** Representative pictures of H&E staining for adipose tissues of the H group. **c** Representative pictures of H&E staining for adipose tissues of the F-0.6 group.

Measurement of Maleic Dialdehyde

Maleic dialdehyde (MDA) was determined with thiobarbituric acid (TBA) using the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Total protein content of the samples was analyzed using Coomassie Blue assay (Nanjing Jiancheng Bioengineering Institute).

Measurement of Infiltration of Polymorphonuclear Cells

Meloperoxidase (MPO) activity was measured to assess the extent of polymorphonuclear cell (PMN) infiltration. The method of assaying MPO activity was according to the guide of the assay kit (Nanjing Jiancheng Bioengineering Co., Ltd., China).

Measurement of Interleukin-1β and Tumor Necrosis Factor Alpha Level

The concentration of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) was determined using a commercial ELISA kit (Shanghai Jinma Biological Technology, Inc., China) following the manufacturer's instruction.

Measurement of Cyclooxygenase-2, Inducible Nitric Oxide Synthase, and ICAM-1 Levels

The procedures were processed according to the protocols recommended for cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and ICAM-1 immunohistochemistry kit (Hengdabaisheng Biotechnology, Beijing, China).

Antioxidant Activities of Fucoxanthin with Undifferentiated PC12 Cells

Cell Culture and Treatment

The PC12 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 6 % FBS, and 6 % horse serum at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. They were plated on poly-D-lysine-coated 96-well plate at a

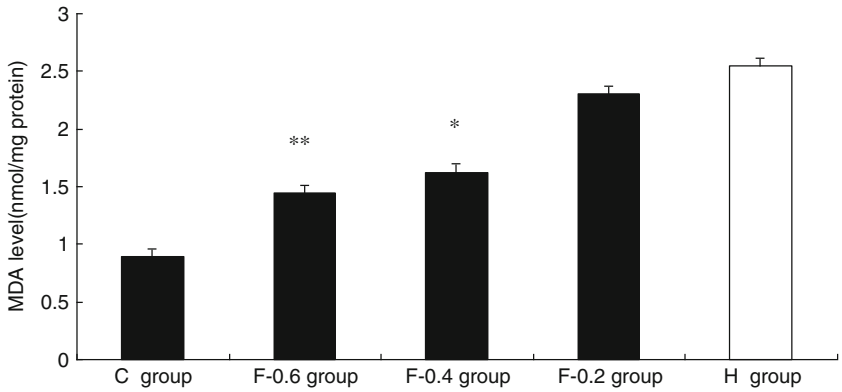

Fig. 4. Effects of fucoxanthin on MDA level. Values are shown as means ± SEM. **P*<0.05 *vs.* H group, ***P*<0.01 *vs.* H group.

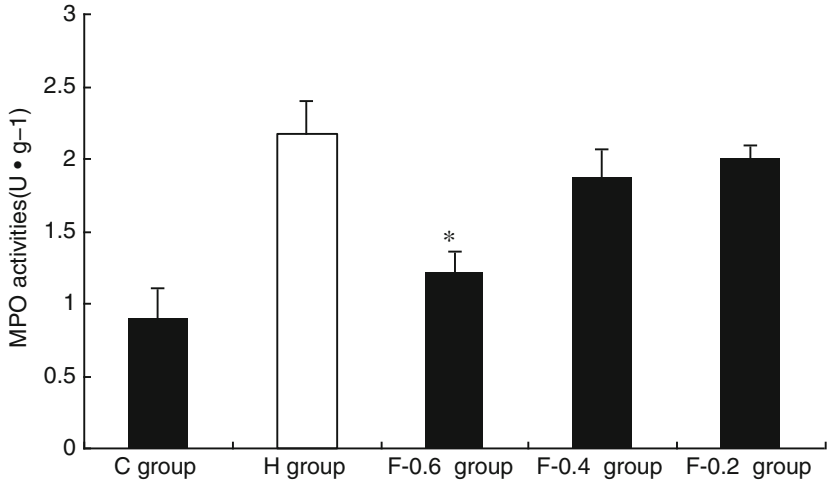

Fig. 5. Effects of fucoxanthin on MPO activities. Values are shown as means ± SEM. *$P<0.05$ *vs.* H group.

density of 2×10^4 cells/well and allowed to adhere for 24 h at 37 °C with complete DMEM. The PC12 cells were then induced to differentiate with 50 ng/mL NGF in serum-free DMEM for 3 days. Thereafter, the culture medium was replaced by fresh serum-free DMEM (without NGF) with or without the sterile fucoxanthin for 2 h.

Measurement of Intracellular Reactive Oxygen Species Formation

After treatments, cells were incubated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 30 min. Fluorescence intensity was measured for 2 h using a fluorometer (FLx800, BioTek Instruments) at 485 and 530 nm λ_{exc} and λ_{em}, respectively.

Enzyme Activity Assays

The supernatant of total cellular extract was used for the assay of glutathione (GSH) content, superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, catalase (CAT) activity, Na^+K^+ATPase activity, and glutathione S transferase (GST) activity.

Statistical Analysis

The data were expressed as mean ± SEM, and results were analyzed by ANOVA followed by Dunnett's *t* test. $P<0.05$ was considered significant.

RESULTS

Initially, we investigated the effects of fucoxanthin on the weights of mice. The body weights of the mice are presented in Fig. 2. Contrasted with the H group, the body weights of mice in the fucoxanthin (0.4 and 0.6)-treated group decreased gradually 2 weeks later. On the contrary, the body weights of mice in the fucoxanthin (0.2)-treated group did not decrease significantly.

The histology of the mammary gland was assessed to quantify the severity of inflammation (Fig. 3). In comparison to the C group (Fig. 3a), a significant increase in mammary gland inflammation was found in the H group mice following 9 weeks of high-sucrose feeding

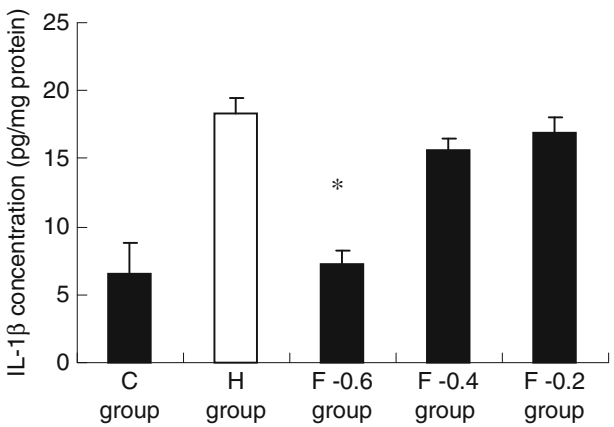

Fig. 6. Effects of fucoxanthin on IL-1β concentration. Values are shown as means ± SEM. *$P<0.05$ *vs.* H group.

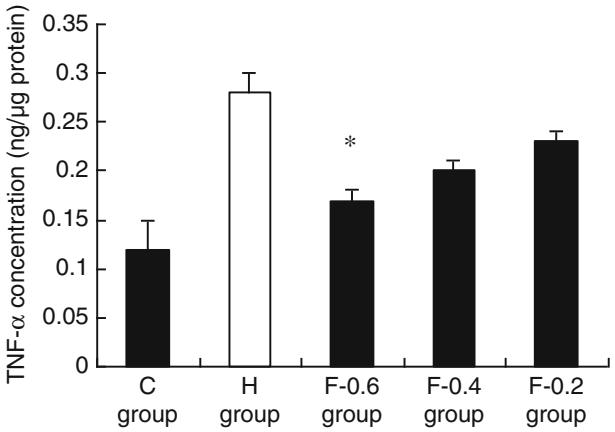

Fig. 7. Effects of fucoxanthin on TNF-α concentration. Values are shown as means ± SEM. *$P<0.05$ *vs.* H group.

(Fig. 3b). Notably, high-fat feeding for 9 weeks followed by fucoxanthin for 5 weeks was associated with a significant decrease in the severity of mammary gland inflammation compared to the H group (Fig. 3c).

The MDA level in the H group was found to be 0.966±0.007 nmol. A significant decrease in the MDA level was observed in the C group, as compared to the H group ($P<0.01$). The levels of MDA decreased after administration of 0.6 mg fucoxanthin ($P<0.01$) and 0.4 mg fucoxanthin ($P<0.05$). However, the same results did not occur in the fucoxanthin (0.2)-treated group (Fig. 4).

The activity of MPO was determined as an indicator of PMN migration. In this study, the MPO activity was 0.90 U/g in C rats and significantly increased in the H group. Treatment with fucoxanthin (0.6 mg) significantly reduced MPO activity (1.30 U/g, $P<0.05$). Treatment with 0.4 and 0.2 mg fucoxanthin reduced MPO activity also. However, it is not significant (Fig. 5).

Figure 6 shows that H diet significantly increased protein concentration of IL-1β in the blood. Treatment of 0.6 mg fucoxanthin decreased the level of IL-1β (13.88 pg/mg) as compared to the H group ($P<0.05$). As shown in Fig. 7, the levels of TNF-α elevated significantly after H diet treatment; 0.6 mg fucoxanthin suppressed this response (0.15 ng/μg, $P<0.05$).

Mice subjected to H diet showed typical markers of inflammation including upregulation of prooxidative enzymes (COX-2 and iNOS) (Table 1). The protein expressions iNOS and COX-2 decreased dose dependently in the fucoxanthin-treated groups (Table 1).

Following, the possible protective effect of them on an oxidative stress model was analyzed with PC12 cells. Exposure to the Fenton reaction led to a significant increase of intracellular reactive oxygen species (ROS) generation. A reduction in ROS formation was observed when cells were incubated with fucoxanthin. This reduction was significant for F-0.6 treatment (Fig. 8).

Spectrophotometric assays revealed that Fenton reaction exposure of 30 min in PC12 cells caused a significant decrease in activity and protein expression of the antioxidant enzymes compared to control cells. F-0.6 and F-0.4 treatments showed a significant ($P<0.05$–0.01) restoration in the level of various enzymes as compared with the Fenton group. However, there was no significant difference between the Fenton and F-0.2 groups (Table 2).

DISCUSSION

Obesity-induced inflammation is characterized by the abnormal production of pro- and anti-inflammatory adipocytokines. It has been found that resident macrophages in adipose tissue are mainly responsible for the

Table 1. Effect of Fucoxanthin on iNOS and COX-2 Protein Production (Number of Immunopositive per Square Millimeter)

Different groups	iNOS	COX-2
C group	11.24±4.90a	10.06±5.06a
H group	60.23±16.31b	70.41±9.20b
F-0.6 group	27.41±1.29a	31.08±5.15a
F-0.4 group	47.41±1.19a	41.08±5.05a
F-0.2 group	56.31±3.99b	53.07±11.25b

The different letters in the same column indicate a statistical difference ($P<0.05$)

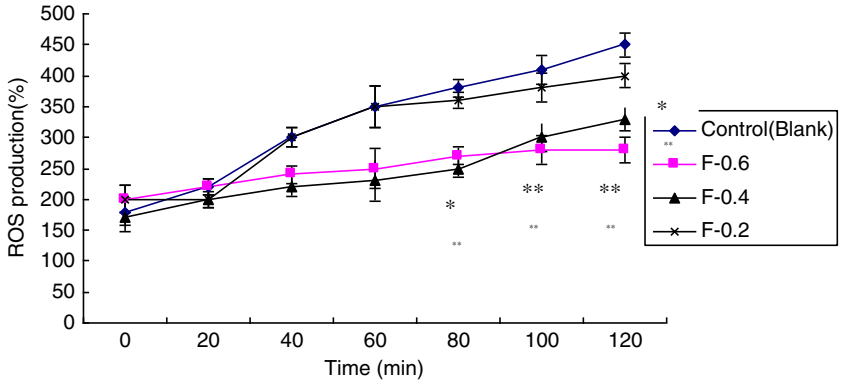

Fig. 8. Effect of fucoxanthin on ROS production in PC12 cells. Results are expressed as mean ± SEM. * $P<0.05$ *vs.* control group, ** $P<0.01$ *vs.* control group.

production of inflammatory cytokines. In mouse models of obesity, inflammatory foci characterized by CLS consisting of dead adipocytes encircled by macrophages were found in the mammary gland. The histology of the mammary gland was assessed to quantify the severity of inflammation.

In the current study, we replicated our initial findings by demonstrating that HFD-induced obesity was associated with histological inflammation and hypertrophy of mammary adipocytes. It is consistent with other studies [12, 13]. Treatment with fucoxanthin significantly suppressed this process, suggesting that the antiobese effect of fucoxanthin may be achieved, at least in part, through blocking necrosis and chronic inflammation in adipose tissues.

High-fat exposure is known to drive the proinflammatory activation of macrophages potentially through multiple major pathways including the production of reactive oxygen [14]. Therefore, the antioxidant effects of fucoxanthin were investigated by measuring MDA levels. As shown in Fig. 4, the MDA levels in the F-0.4 and F-0.6 groups were significantly lower than those in the saline group ($P<0.05$ and $P<0.01$, respectively). The activity of MPO was determined as an indicator of PMN migration. In this study, the MPO activity was relatively low in the C group and significantly increased in the H group. Treatment with 0.6 mg fucoxanthin/kg weight resulted in a substantial decrease in the extent of PMN infiltration in the obesity-induced inflammation. High levels of oxidative stress are common in many diseases. Carotenoids have been implicated as important dietary nutrients with antioxidant potential [15]. As a carotenoid, fucoxanthin is a powerful antioxidant that protects cells from oxidative stress damage.

In obese adipose, chronic inflammation ensues with proinflammatory release of TNF-α and IL-1β [16, 17]. IL-1β interaction with adipocyte IL-1 receptor promotes disruption of the adipocyte insulin signaling pathway, thus contributing to decreased insulin sensitivity [18]. Several studies also showed that TNF-α plays an important role in the development of obesity-induced insulin resistance in murine models by impairing insulin signaling, particularly in adipocytes [19, 20]. Our results show that 0.6 mg fucoxanthin treatment decreased the level of IL-1β and significantly suppressed the elevated levels of TNF-α.

Table 2. Effect of Fucoxanthin on Antioxidant Enzyme Activity

Different groups	GSH (nmol/mL)	SOD (IU/mg protein)	GPx	GR	GST	CAT	Na^+K^+ATPase
F-0.2 group	1.401±0.022*	1.82±1.22	8.10±0.32	24.31±2.02	10.60±0.66	4.88±0.32	3.00±0.31
F-0.4 group	1.500±0.033*	1.33±3.21*	14.11±1.12**	30.21±6.03**	17.66±2.33**	6.66±0.44*	4.11±0.21*
F-0.6 group	1.830±0.011*	4.33±0.56**	16.00±2.23**	36.56±2.56**	17.44±1.23**	7.22±0.32**	4.56±0.60*
Fenton group	1.112±0.011	2.01±1.41	7.89±0.33	21.11±2.23	9.07±1.11	4.66±0.10	2.22±0.20

Values are shown as means ± SEM
*$P<0.05$ (*vs.* Fenton group); **$P<0.01$ (*vs.* Fenton group)

The present work was also undertaken to evaluate the anti-inflammatory effects of fucoxanthin on COX-2 expression, iNOS expression, and level of ICAM-1. Inducible NOS is induced in response to inflammatory-like stimuli and is capable of sustained production of high levels of NO that predominate during inflammation [21]. The excessive or inappropriate production of NO can damage tissue through the superoxide anion (O_2^-) [22]. The protein expressions of iNOS decreased dose dependently in the fucoxanthin-treated groups. NO also activates COX enzymes, leading to a marked increase in PGE2 production [23]. COX-2 is primarily responsible for increased PGE2 production during inflammation. In the present work, F-0.6 treatment significantly decreased the expression of COX-2 protein in obesity-induced inflammation ($P<0.05$). The results of this work showed that fucoxanthin prevents inflammation and insulin resistance also by inhibiting nitric oxide (NO) and PGE2 production through the downregulation of iNOS and COX-2 mRNA expression as well as adipocytokine production.

Recent studies by Cruz *et al.* and Bulua *et al.* demonstrate that the promoted inflammation is correlated with increased ROS production [24, 25]. Following, the possible protective effect of fucoxanthin on an oxidative stress model was analyzed in PC12 cells. A reduction in ROS formation was observed when cells were incubated with fucoxanthin. One of the possible preventive mechanisms of fucoxanthin against oxidative stress consists of removing the ROS excess. At the same time, a significant increase in both protein expression and activity of antioxidant enzymes was detected when PC12 cells were pretreated with fucoxanthin. These results provide further evidences to support those previous findings on potential benefits of fucoxanthin to block HFD-induced obesity and insulin resistance under oxidative stress situation.

CONCLUSION

Our data have shown several findings on the anti-inflammatory and antioxidant functions of fucoxanthin. Fucoxanthin prevents inflammation and insulin resistance by inhibiting upregulation of inflammatory cytokines IL-1β and TNF-α, blocking inflammation-related events (MDA and MPO) and expressions of prooxidative enzymes such as COX-2 and iNOS, as well as removing the ROS excess. These beneficial effects were correlated with a blockade of macrophage infiltration and chronic inflammation in adipose tissue.

ACKNOWLEDGMENTS

This work was supported by the Jinan Municipal Science and Technology Planning Project (grant no. 201102037) and the S&T Plan Projects of Shandong Provincial Education Department (grant no. J11LC12).

REFERENCES

1. Bruun, J.M., A.S. Lihn, S.B. Pedersen, and B. Richelsen. 2005. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): Implication of macrophages resident in the AT. *Journal of Clinical Endocrinology and Metabolism* 90: 2282–2289.
2. Christiansen, T., B. Richelsen, and J.M. Bruun. 2005. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *International Journal of Obesity (London)* 29: 146–150.
3. Hotamisligil, G.S., and E. Erbay. 2008. Nutrient sensing and inflammation in metabolic diseases. *Nature Reviews Immunology* 8: 923–934.
4. Makowski, L., and G.S. Hotamisligil. 2004. Fatty acid binding proteins—The evolutionary crossroads of inflammatory and metabolic responses. *Journal of Nutrition* 134: 2464S–2468S.
5. Ferrante Jr., A.W. 2007. Obesity-induced inflammation: A metabolic dialogue in the language of inflammation. *Journal of Internal Medicine* 262: 408–414.
6. Shoelson, S.E., and J. Lee. 2006. Goldfine AB: Inflammation and insulin resistance. *Journal of Clinical Investigation* 116: 1793–1801.
7. Dembitsky, V.M., and T. Maoka. 2007. Allenic and cumulenic lipids. *Progress in Lipid Research* 46: 328–375.
8. Yan, X., Y. Chuda, M. Suzuki, and T. Nagata. 1999. Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed. *Bioscience, Biotechnology, and Biochemistry* 63: 605–607.
9. Woo, M.N., S.M. Jeon, H.J. Kim, M.K. Lee, S.K. Shin, Y.C. Shin, Y.B. Park, and M.S. Choi. 2010. Fucoxanthin supplementation improves plasma and hepatic lipid metabolism and blood glucose concentration in high-fat fed C57BL/6N mice. *Chemico-Biological Interactions* 186: 316–322.
10. Jeon, S.M., H.J. Kim, M.N. Woo, M.K. Lee, Y.C. Shin, Y.B. Park, and M.S. Choi. 2010. Fucoxanthin-rich seaweed extract suppresses body weight gain and improves lipid metabolism in high-fat-fed C57BL/6J mice. *Biotechnology Journal* 5: 961–969.
11. Maeda, H., M. Hosokawa, T. Sashima, and K. Miyashita. 2007. Dietary combination of fucoxanthin and fish oil attenuates the weight gain of white adipose tissue and decreases blood glucose in obese/diabetic KK-Ay mice. *Journal of Agricultural and Food Chemistry* 55: 7701–7706.
12. Cinti, S., G. Mitchell, G. Barbatelli, I. Murano, E. Ceresi, E. Faloia, *et al*. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of Lipid Research* 46(11): 2347–2355.
13. Murano, I., G. Barbatelli, V. Parisani, C. Latini, G. Muzzonigro, M. Castellucci, *et al.* 2008. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *Journal of Lipid Research* 49(7): 1562–1568.
14. Sheppard, F.R., M.R. Kelher, E.E. Moore, N.J. McLaughlin, A. Banerjee, and C.C. Silliman. 2005. Structural organization of the neutrophil NADPH oxidase: Phosphorylation and translocation during priming and activation. *Journal of Leukocyte Biology* 78: 1025–1042.

15. Sachindra, N.M., E. Sato, H. Maeda, M. Hosokawa, Y. Niwano, M. Kohno, and K. Miyashita. 2007. Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites. *Journal of Agricultural and Food Chemistry* 55: 8516–8522.
16. Xu, H., *et al.* 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *Journal of Clinical Investigation* 112: 1821–1830.
17. Neels, J.G., and J.M. Olefsky. 2006. Inflamed fat: What starts the fire? *Journal of Clinical Investigation* 116: 33–35.
18. Wen, H., *et al*. 2011. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nature Immunology* 12: 408–415.
19. Ruan, H., N. Hacohen, T.R. Golub, L. Van Parijs, and H.F. Lodish. 2002. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: Nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes* 51: 1319–1336.
20. Uysal, K.T., S.M. Wiesbrock, and G.S. Hotamisligil. 1998. Functional analysis of tumor necrosis factor (TNF) receptors in TNF-alpha-mediated insulin resistance in genetic obesity. *Endocrinology* 139: 4832–4838.
21. Clancy, R.M., A.R. Amin, and S.B. Abramson. 1998. The role of nitric oxide in inflammation and immunity. *Arthritis and Rheumatism* 41: 1141–1151.
22. Yaren, H., H. Mollaoglu, B. Kurt, *et al.* 2007. Lung toxicity of nitrogen mustard may be mediated by nitric oxide and peroxynitrite in rats. *Research in Veterinary Science* 83: 116–122.
23. Mandai, M., N. Yoshimura, M. Yoshida, M. Iwaki, and Y. Honda. 1994. The role of nitric oxide synthase in endotoxin-induced uveitis: Effects of N^G-nitro-L-arginine. *Investigative Ophthalmology & Visual Science* 35: 3673–3680.
24. Cruz, C.M., A. Rinna, H.J. Forman, A.L. Ventura, P.M. Persechini, and D.M. Ojcius. 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *Journal of Biological Chemistry* 282(5): 2871–2879.
25. Bulua, A.C., A. Simon, R. Maddipati, M. Pelletier, H. Park, K.Y. Kim, *et al.* 2011. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *Journal of Experimental Medicine* 208(3): 519–533.