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ORIGINAL CONTRIBUTION

Fucoxanthin inhibits lipopolysaccharide-induced inflammation and oxidative stress by activating nuclear factor E2-related factor 2 via the phosphatidylinositol 3-kinase/AKT pathway in macrophages

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Abstract

Purpose Anti-inflammatory and antioxidant effects of fucoxanthin (FCX), a xanthophyll carotenoid, have been suggested. However, underlying mechanisms are elusive. The objective of this study was to elucidate the mechanisms by which FCX and its metabolites inhibit lipopolysaccharide (LPS)-induced inflammation and oxidative stress in macrophages.

Methods The effects of the FCX on mRNA and protein expression of pro-inflammatory cytokines and antioxidant genes, and reactive oxygen species (ROS) accumulation were determined in RAW 264.7 macrophages. A potential role of FCX in the modulation of phosphatidylinositol 3-kinase (PI3K)/AKT/nuclear E2-related factor 2 (NRF2) axis was evaluated.

Results FCX significantly decreased LPS-induced interleukin (*Il*)6, *Il1b*, and tumor necrosis factor α (*Tnf*) mRNA abundance and TNFα secretion. FCX attenuated LPS or tert-butyl-hydroperoxide-induced ROS accumulation with concomitant increases in the expression of antioxidant enzymes. Also, trolox equivalent antioxidant capacity assay demonstrated that FCX had a potent free radical scavenging property. FCX markedly increased nuclear translocation of NRF2 in LPS-treated macrophages, consequently inducing its target gene expression. Interestingly, the effect of FCX on NRF2 nuclear translocation was noticeably diminished by LY294002, an inhibitor of PI3K, but not by inhibitors of mitogen-activated protein kinases. Phosphorylation of AKT, a downstream element of PI3K, was also markedly increased by FCX. FCX metabolites, such as fucoxanthinol and amarouciaxanthin A, significantly attenuated LPS-induced ROS accumulation and pro-inflammatory cytokine expression.

Conclusion FCX exerts anti-inflammatory and antioxidant effects by the activation of NRF2 in the macrophages activated by LPS, which is mediated, at least in part, through the PI3K/AKT pathway.

Keywords Fucoxanthin · Xanthophyll · Inflammation · Oxidative stress · NRF2 · PI3K · Macrophages

Abbreviations

ABTS	2,2′-Azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid)
ACXA	Amarouciaxanthin A
ARE	Antioxidant response element
BMDM	Bone marrow-derived macrophages
Cat	Catalase
FCN	Fucoxanthinol
FCX	Fucoxanthin
FAK	Focal adhesion kinase
Gpx1	Glutathione peroxidase 1
Hmox1	Heme oxygenase-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
NF-κB	Nuclear factor κB
NRF2	Nuclear E2-related factor 2
PI3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
Sod1	Superoxide dismutase 1
TAC	Total antioxidant capacity
t-BHP	Tert-butyl-hydroperoxide
TEAC	Trolox equivalent antioxidant capacity
TNFα	Tumor necrosis factor α

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Introduction

Chronic inflammation and oxidative stress are closely related to the pathogenesis of obesity-induced chronic diseases, such as cardiovascular disease, insulin resistance, non-alcoholic fatty liver, and type 2 diabetes [1–3]. Macrophages play a crucial role in the inflammatory responses by secreting pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 [4]. Also, activated macrophages produce reactive oxygen species (ROS), triggering oxidative stress and inflammation [5]. Therefore, inhibition of intracellular ROS accumulation in macrophages can ameliorate the pro-inflammatory pathways in macrophages [6].

Nuclear factor E2-related factor 2 (NRF2) is vital for the endogenous antioxidant defense as it regulates the transcription of several antioxidant genes [7, 8]. Also, the activation of NRF2 inhibits pro-inflammatory signals by preventing the activation of nuclear factor κB (NF-κB) [6]. Under oxidative stress conditions, Kelch-like ECH-associated protein 1 (Keap1) is released from NRF2, allowing the nuclear translocation of NRF2 to induce the expression of antioxidant genes [8]. The translocation of NRF2 to the nucleus is known to be regulated by kinases, such as phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) [6, 9]. NRF2 activation has been shown to be beneficial in alleviating inflammation-related pathogenesis, such as rheumatoid arthritis, gastritis, colitis, atherosclerosis, and intracerebral bleeding [10]. Therefore, NRF2 activators may be considered an agent for the prevention or treatment of inflammation-mediated diseases.

Fucoxanthin (FCX) is a xanthophyll carotenoid abundant in edible brown seaweed. Studies have shown that FCX has antioxidant [11, 12] and anti-inflammatory properties [13–15]. We recently reported that FCX reduced transforming growth factor β1-induced ROS accumulation by decreasing the expression of nicotinamide adenine dinucleotide phosphate oxidase 4, an ROS-producing enzyme, in hepatic stellate cells, supporting the anti-fibrogenic effect of FCX [16]. FCX showed an anti-inflammatory property in mice fed a high-fat diet with decreased serum levels of IL-1β and TNFα [17]. FCX inhibited lipopolysaccharide (LPS)-induced pro-inflammatory cytokines in BV-2 microglial cells through the inhibition of protein kinase B (AKT)/NF-κB and MAPKs/activator protein 1 (AP-1) pathways [18]. Also, FCX decreased the production of pro-inflammatory mediators by inhibiting NF-κB and MAPKs signaling pathways in LPS-induced RAW 264.7 macrophages [13]. However, the effects of FCX on NRF2-mediated anti-inflammatory and antioxidant responses in macrophages remain unknown. Furthermore, as FCX is metabolized to fucoxanthinol (FCN) in the gastrointestinal tract and further to amarouciaxanthin A (ACXA) in the liver [19], it is important to understand bioactivities of these FCX metabolites in the inflammatory pathways in macrophages. Therefore, in the present study, we investigated whether FCX and its metabolites inhibit LPS-induced inflammation and oxidative stress by the modulation of NRF2 in macrophages.

Materials and methods

Cell culture and treatment

RAW 264.7 macrophages (ATCC, Manassas, VA, USA) were cultured as we previously described [20]. FCX (≥ 95%), FCN (≥ 97%), and ACXA (≥ 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and prepared for in vitro experiments as we previously described [16]. Macrophages were treated with FCX (5 μM), FCN (0.05 μM), or ACXA (0.1 μM) for 12 h, and then stimulated by 100 ng/ml of LPS (Sigma-Aldrich) for 6 h with or without FCX, FCN, or ACXA. Cells and conditioned medium were collected to measure pro-inflammatory cytokine expression and secretion, respectively. All experiments were performed in the dark to prevent any light-induced degradation.

FCX cytotoxicity test

RAW 264.7 macrophages were treated with FCX at 0–15 μM concentrations for 24 h. The cell viability was assessed using a Cell Counting Kit-8 (Dojindo Inc., Rockville, MD, USA) according to the manufacturer's instructions. Cell viability was calculated as percentages of the control.

Cytokine measurements

The conditioned medium was collected after RAW 264.7 macrophages were treated with FCX as described above and centrifuged at 12,000 × *g* for 5 min to remove any cell debris or dead cells. TNFα secretion was quantified by an enzyme-linked immunosorbent assay using a TNFα mouse uncoated ELISA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Cellular ROS measurement

RAW 264.7 macrophages were pretreated with or without FCX (5 μM), FCN (0.1 μM), or ACXA (0.2 μM) for 24 h and then stimulated with LPS (100 ng/mL) for additional 24 h in the absence or presence of FCX, FCN, or ACXA. Tert-butyl-hydroperoxide (t-BHP; Acros Organics, Geel, Belgium, USA) was used as another ROS generator. RAW

264.7 macrophages were pretreated with or without 5 μM of FCX for 12 h and then stimulated with 200 μM t-BHP for 2 h in the absence or presence of 5 μM of FCX. Cellular ROS levels were measured in RAW 264.7 macrophages as previously described using 2′,7′-dicholorofluorescin (DCFH; Sigma-Aldrich) [21].

Measurement of total antioxidant capacity

The total antioxidant capacity (TAC) of FCX was determined by Trolox equivalent antioxidant capacity (TEAC) assay with the radical action of 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Sigma-Aldrich). Briefly, ABTS radical cation ($ABTS^{\cdot+}$) solution was prepared by mixing both 14 mM ABTS solution and 4.9 mM potassium persulfate solution in equal quantities and incubated for 16 h at room temperature in the dark. The $ABTS^{\cdot+}$ solution was diluted with ethanol to adjust the absorbance to 0.700 ± 0.030 at 750 nm. The absorbances of a mixture of 100 μl FCX at difference concentration (31.3–2000 μM) and 900 μl ABTS solution were measured at 750 nm after 6 min-reaction. The ABTS radical scavenging activity was calculated using a Trolox (9.4–300 μM, Sigma-Aldrich) standard curve. TAC is expressed as μM of Trolox equivalent (TE)/μM of FCX.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (ThermoFisher Scientific, Waltham, MA), and cDNA synthesis and qRT-PCR analysis using the SYBR Green and CFX96 Real-Time system (Bio-Rad, Hercules, CA) were performed as previously described [22]. Primer sequences are listed in Supplementary Table 1.

Western blot analysis

RAW 264.7 macrophages were preincubated with FCX (5 μM) for 24 h and subsequently for additional 3 h with FCX replenishment. The cells were stimulated with LPS (100 ng/ml) for 6 or 12 h in the absence or presence of FCX (5 μM). Subsequently, total cell lysates or cytoplasmic and nuclear fractions using a Cayman nuclear extraction kit (Ann Arbor, MI, USA) were prepared. Western blot analysis was performed as we previously described [22, 23].

The following inhibitors were purchased form Cayman Chemical: LY294002, a PI3K inhibitor; PD98059, an extracellular signal-regulated kinase (ERK) 1/2 inhibitor; SP600125, a c-Jun N-terminal kinase (JNK) 1/2 inhibitor; and SB203580, a p38 inhibitor. RAW 264.7 macrophages were treated with FCX (5 μM) for 24 h followed by additional FCX (5 μM) treatment for 3 h. Subsequently, the cells were activated by 100 ng/ml LPS in the presence of 10 μM of LY294002 or MAPK inhibitor (10 μM of PD98059, SP600125, or SB203580) for 24 h with or without FCX, after which Western blot was conducted. The following antibodies were used: NRF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TATA-binding protein (TBP; Cell Signaling Technologies, Danvers, MA), phospho-AKT (Cell Signaling Technologies), and total-AKT (Cell Signaling Technologies). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) was used as a loading control.

Statistical analysis

One-way analysis of variance (ANOVA) with Newman–Keuls post hoc test or unpaired *t* test was conducted to determine significant differences between groups using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). All data were considered statistically significant at p value < 0.05. All values are presented as mean ± SEM.

Results

FCX decreased LPS-induced expression and secretion of pro-inflammation cytokines in RAW 264.7 macrophages

When cytotoxicity of FCX was tested at a range of 0–15 μM in RAW 264.7 macrophage, cells treated with 5 μM FCX were ~ 90% viable (Fig. 1a). Thus, we used 5 μM of FCX for the following experiments. In a time-course experiment, the expression of *Il1b* and *Il6* reached the highest levels at 6 h of LPS treatment, and FCX significantly reduced the mRNA levels at 6 and 12 h (Fig. 1b). While the expression of *Tnf* was highest 3 h after LPS treatment, at which it was significantly decreased by FCX. FCX significantly reduced the expression levels of *Il1b* and *Tnf* as well as the secretion of TNFα in the medium when RAW 264.7 macrophages were activated by LPS for 6 h (Fig. 1c, d).

FCX inhibited LPS or t-BHP-induced ROS accumulation by increasing antioxidant gene expression in RAW 264.7 macrophages

Oxidative stress by excessive ROS generation in macrophages is closely linked to the induction of pro-inflammatory gene expression [6]. FCX is known to have a potent antioxidant effect [11, 16]. We used LPS and t-BHP to evaluate the effect of FCX on cellular ROS accumulation in RAW 264.7 macrophages. LPS significantly increased intracellular ROS levels, which was significantly diminished by FCX (Fig. 2a). Also, elevated cellular ROS levels by t-BHP were almost completely abolished

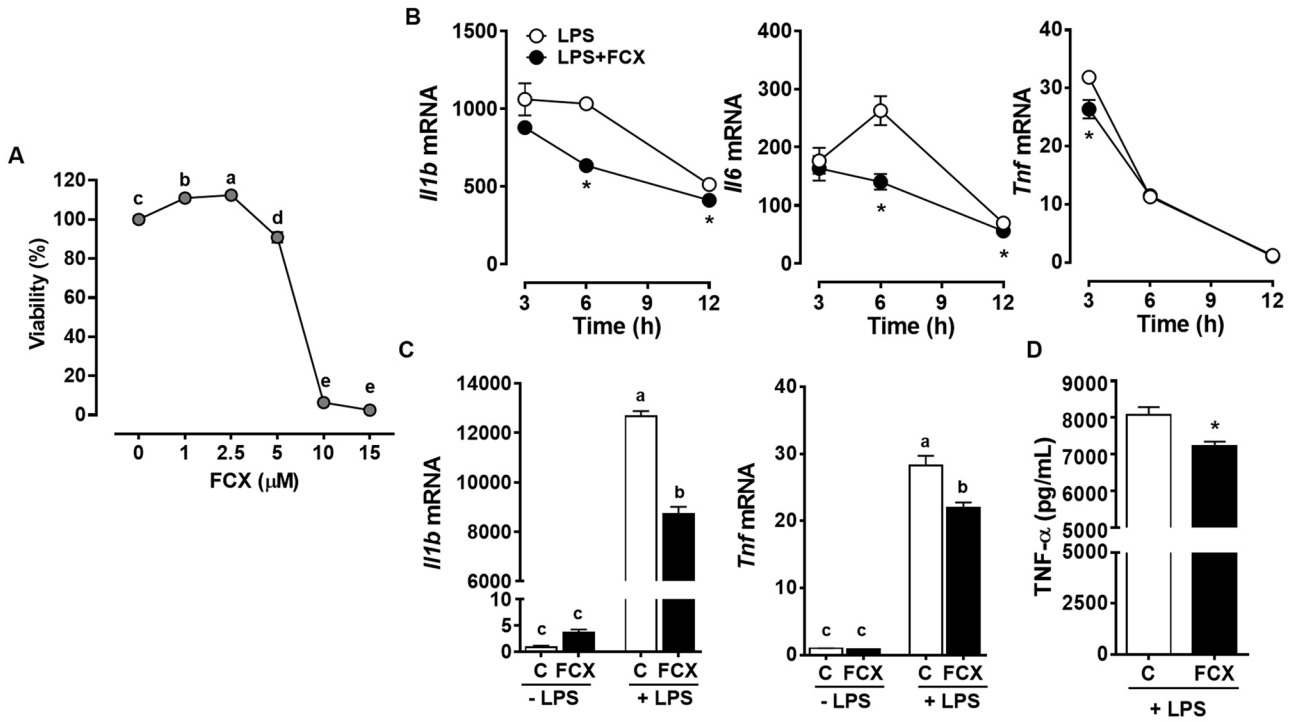

Fig. 1 The inhibitory effects of FCX on the expression and secretion of pro-inflammatory cytokines in LPS-induced RAW 264.7 macrophages. **a** Cytotoxicity of FCX in RAW 264.7 macrophages. Cells were treated with 0, 1, 2.5, 5, 10, and 15 μM of FCX for 24 h to measure the cell viability. FCX concentrations with a different letter are significantly different ($P < 0.05$). **b** Cells were pretreated with FCX (5 μM) for 24 h, and subsequently stimulated by LPS (100 ng/mL) for 3, 6, and 12 h with or without FCX for gene analysis. *Significantly different from control ($P < 0.05$). **c**, **d** Cells were pretreated with FCX (5 μM) for 12 h, and subsequently stimulated by LPS (100 ng/mL) for 6 h with or without FCX for gene analysis. C, control. Bars with a different letter are significantly different ($P < 0.05$). Data are presented as Mean ± SEM

by FCX to the basal levels (Fig. 2b). We further determined the TAC of FCX using a TEAC assay. TEAC values of FCX ranged from 21.3 to 288.9 μM TE/μM FCX (Fig. 2c). FCX at 100 μM showed TEAC values of 57.6 TE μM.

LPS significantly reduced the expression of antioxidant genes, such as superoxide dismutase (*Sod1*), glutathione peroxidase 1 (*Gpx1*), *Gpx4*, and catalase (*Cat*), which were significantly increased by FCX (Fig. 3). The expression of *Sod2* was significantly increased by LPS stimulation, while FCX further increased *Sod2* expression levels. However, FCX did not alter the mRNA abundance of all the antioxidant genes in unstimulated RAW 264.7 macrophages.

FCX-stimulated *Nfe2l2* expression and NRF2 nuclear translocation in LPS-induced RAW 264.7 macrophages

NRF2 is known to play a protective role against oxidative stress and inflammation through the induction of antioxidant enzymes [24]. LPS significantly decreased mRNA levels of *Nfe2l2*, the gene name for NRF2, while it was significantly increased by FCX (Fig. 4a). The expression of heme oxygenase-1 (*Hmox1*), an NRF2 target gene, was significantly increased by LPS, which was further elevated by FCX. As NRF2 nuclear translocation is a crucial event for NRF2 activation [25], we determined nuclear protein levels of NRF2. While the nuclear translocation of NRF2 by LPS was evident with LPS stimulation for 6 and 12 h, FCX further increased NRF2 protein in the nucleus at both time points (Fig. 4b).

PI3K/AKT signaling pathway mediated FCX-stimulated NRF2 nuclear translocation in LPS-induced RAW 264.7 macrophages

PI3K and MAPKs are known to play a role in the nuclear translocation of NRF2 [26]. To gain insight into the role of the kinases in increased NRF2 nuclear translocation by FCX, we utilized specific inhibitors of PI3K and MAPK pathways. LPS stimulation increased the nuclear levels of NRF2, which was further elevated by FCX (Fig. 5a). Interestingly, LY294002, an inhibitor of PI3K, noticeably abolished the effect of FCX on NRF2 nuclear translocation, but other MAPK inhibitors, such as PD98059, SP600125, and SB203580, showed minimal effects. While LPS increased nuclear NRF2 protein by threefold, LY294002 inhibitor alone decreased the protein level by ~ 40% (Fig. 5b). When cells were treated with LPS

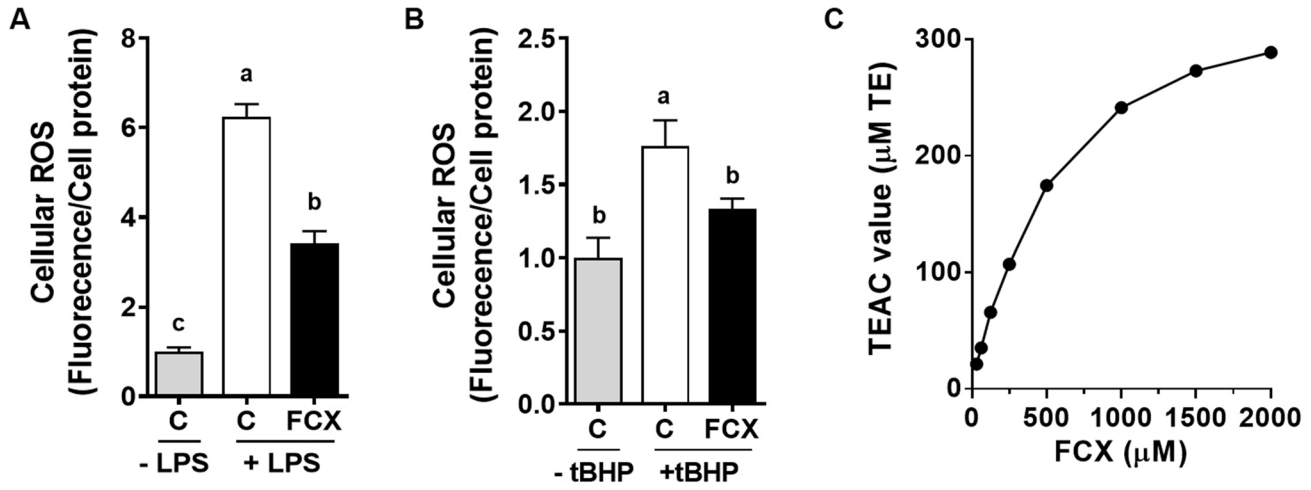

Fig. 2 The inhibitory effects of FCX on cellular ROS accumulation in RAW 264.7 macrophages and TEAC values of FCX. **a** Cells were pretreated with FCX (5 μM) for 24 h, and subsequently stimulated by LPS (100 ng/mL) for 24 h to measure ROS accumulation. **b** Cells were pretreated with FCX (5 μM) for 12 h, and subsequently stimulated by t-BHP (200 μM) for 2 h with or without FCX to measure ROS accumulation. C, control. **c** TAC of FCX was determined using TEAC assay with ABTS radical action. Bars with a different letter are significantly different ($P < 0.05$). Data are presented as Mean ± SEM

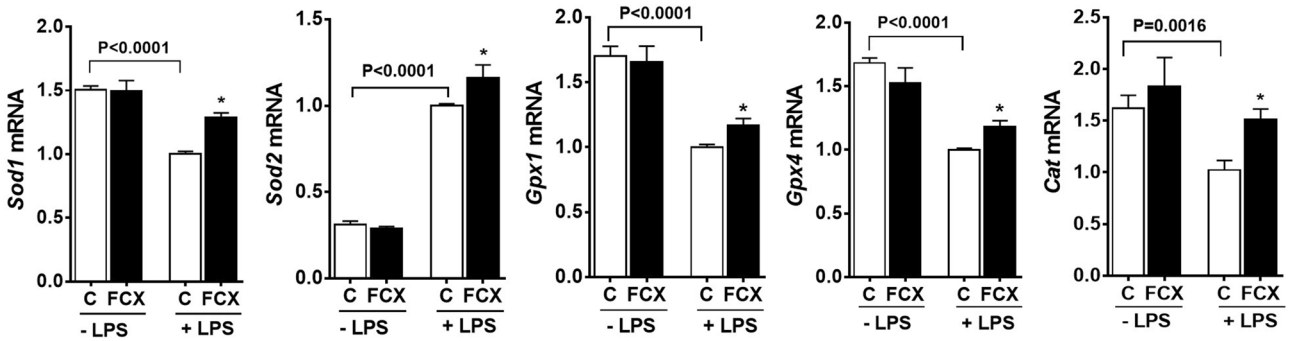

Fig. 3 The effects of FCX on the expression of antioxidant enzymes in RAW 264.7 macrophages. Cells were pretreated with FCX (5 μM) for 12 h, and subsequently stimulated by LPS (100 ng/mL) for 6 h with or without FCX for genes analysis. *Significantly different from control ($P < 0.05$). C, control. Bars with a different letter are significantly different ($P < 0.05$). Data are presented as Mean ± SEM

and FCX together, nuclear NRF2 protein was higher than LPS alone, which was diminished by more than twofold in the presence of LY294002. PI3K activation induces phosphorylation of AKT, which is an upstream kinase of NRF2 [27]. LPS markedly increased phosphorylation of AKT at 12 h, which was further elevated by FCX (Fig. 5c).

FCN and ACXA repressed the LPS-induced ROS accumulation and pro-inflammatory gene expression in RAW 264.7 macrophages

As FCX is metabolized into FCN and ACXA [19], we determined the effects of FCN and ACXA on intercellular ROS accumulation and pro-inflammatory gene expression. Treatment concentrations of FCN and ACXA were determined based on circulating concentrations of FCN and ACXA in blood after the consumption of an FCX-containing diet in mice [28, 29]. Elevated ROS levels by LPS were significantly reduced by both FCN and ACXA (Fig. 6a). LPS significantly increased *Il1b* mRNA levels, which were significantly reduced by FCN and ACXA (Fig. 6b). While ACXA significantly decreased LPS-induced *Tnf* mRNA level, FCN did not alter the gene expression.

Discussion

Chronic low-grade inflammation and oxidative stress are underlying causes of obesity-related metabolic diseases [30]. It is essential to identify antioxidant and anti-inflammatory

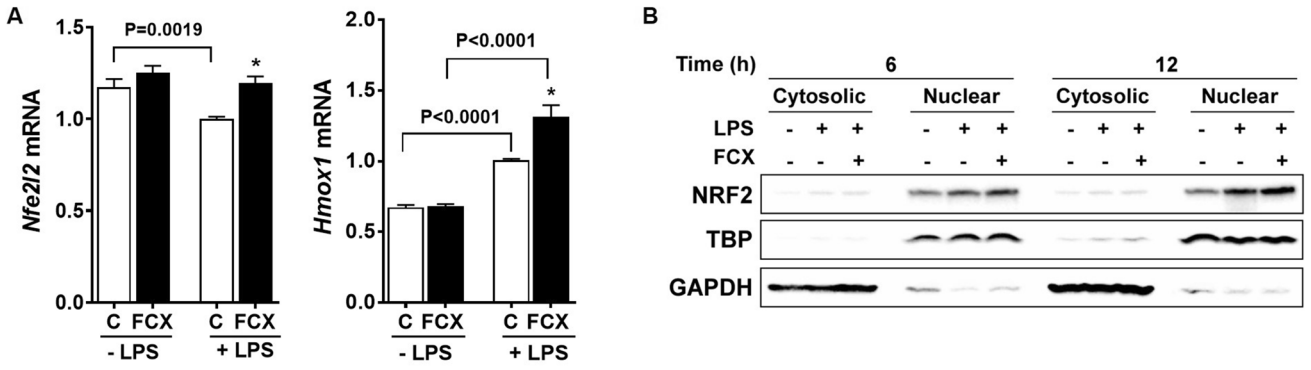

Fig. 4 The effect of FCX on *Nfe2l2* gene expression and nuclear translocation of NRF2 in RAW 264.7 macrophages. **a** Cells were pretreated with FCX (5 μM) for 12 h, and subsequently stimulated by LPS (100 ng/mL) for 6 h with or without FCX for gene analysis. *Significantly different from control ($P < 0.05$). Data are presented as Mean ± SEM. C, control. **b** Cells were pretreated with FCX (5 μM) for 24 h, followed by additional FCX (5 μM) treatment for 3 h and the stimulation by LPS (100 ng/mL) for 6 or 12 h with or without FCX. GAPDH and TBP were used for the purity of cytoplasmic and nuclear fractions, respectively

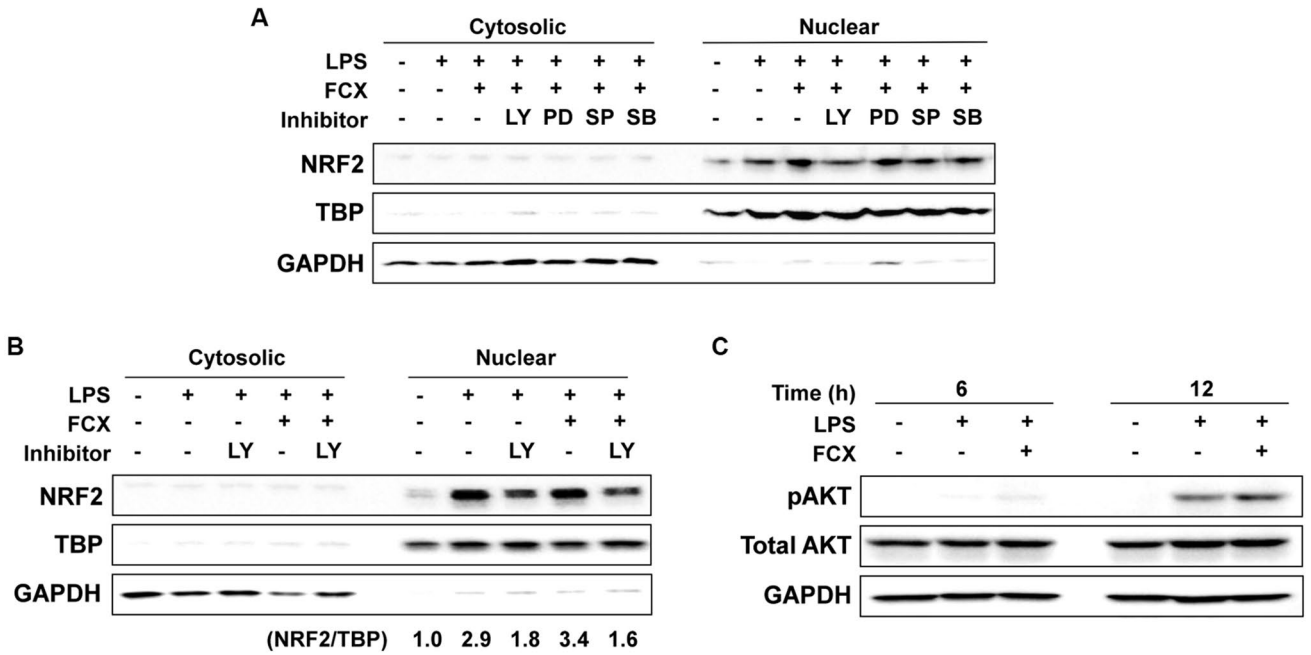

Fig. 5 The effect of PI3K and MAPKs inhibitors on FCX-stimulated NRF2 nuclear translocation in RAW 264.7 macrophages. **a**, **b** Cells were pretreated with FCX (5 μM) for 24 h, followed by additional FCX (5 μM) treatment for 3 h and the stimulation by LPS (100 ng/mL) and inhibitors (10 μM) for 12 h with or without FCX. GAPDH and TBP were used for the purity of cytoplasmic and nuclear fractions, respectively. A representative blot image is shown. LY, LY294002 as PI3K inhibitor; PD, PD98059 as ERK1/2 inhibitor; SP, SP600125 as JNK1/2 inhibitor; SB, SB203580 as p38 inhibitor. Densitometry analysis was conducted for nuclear NRF2 contents using TBP as a loading control, and the values are relative to the control without LPS. **c** Cells were pretreated with FCX (5 μM) for 24 h, followed by additional FCX (5 μM) treatment for 3 h and the stimulation by LPS (100 ng/mL) for 12 h with or without FCX. GAPDH was used as a loading control

components for the prevention or treatment of chronic inflammatory diseases. Studies have demonstrated that FCX exerts antioxidant properties [11, 12, 31] and inhibits pro-inflammatory cytokine production in macrophages [13–15]. However, the effect of FCX on the modulation of the NRF2 pathway to exert antioxidant and anti-inflammatory functions in macrophages has not been determined. In the present study, we found that the inhibitory effect of FCX on inflammation and oxidative stress is attributed, at least in part, to the increased nuclear translocation of NRF2 via the

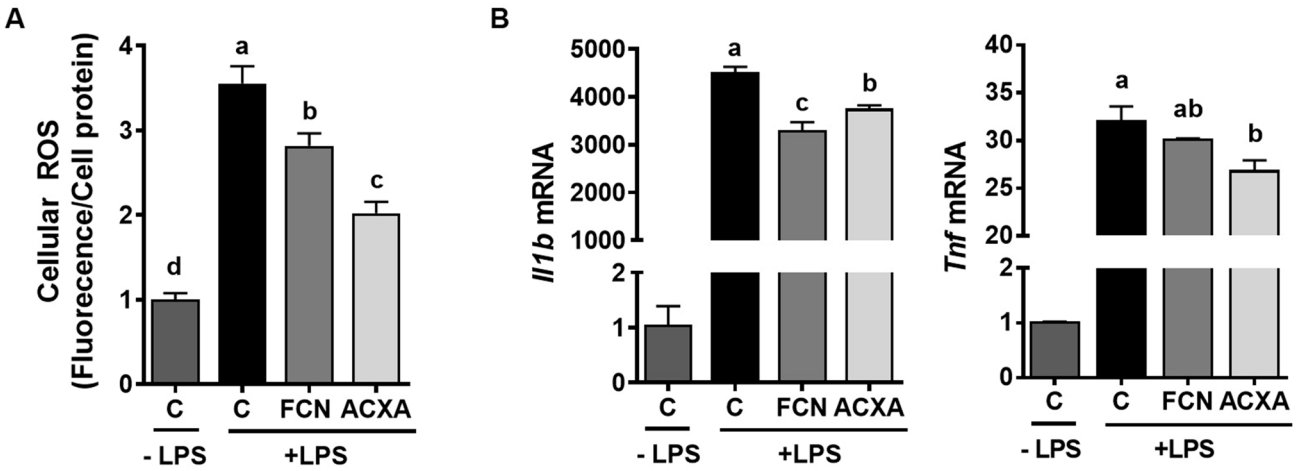

Fig. 6 The inhibitory effect of FCN and ACXA on cellular ROS accumulation and the expression of pro-inflammatory cytokines in LPS-induced RAW 264.7 macrophages. **a** Cells were pretreated with FCN (0.1 μM) or ACXA (0.2 μM) for 24 h, and subsequently stimulated by LPS (100 ng/mL) for 24 h with or without FCN or ACXA to measure ROS accumulation. C, control. **b** Cells were pretreated with FCN (0.05 μM) or ACXA (0.1 μM) for 12 h, and subsequently stimulated by LPS (100 ng/mL) for 6 h with or without FCN or ACXA for gene analysis. Bars with a different letter are significantly different ($P < 0.05$). Data are presented as Mean ± SEM

modulation of the PI3K/AKT signaling pathway in LPS-activated macrophages. Importantly, we also demonstrated, for the first time, FCX metabolites, including FCN and ACXA, reduced LPS-induced ROS accumulation, and pro-inflammatory gene expression in macrophages.

Excessive ROS lead to oxidative stress, characterized by oxidative DNA damage, oxidation of amino acids and lipid peroxidation, which can trigger inflammatory responses [32, 33]. LPS, a major component of bacterial cell walls, induces the generation of ROS while stimulating pro-inflammatory responses in the macrophages [34]. Also, hydrogen peroxides are known to cause cell death and oxidative stress [35]. In the present study, we found that FCX inhibited LPS or t-BHP-induced intercellular ROS accumulation in macrophages. Also, FCX at 100 μM had TEAC values of 57.6 TE μM by scavenging ABTS free radicals. Carotenoids, such as lycopene, β-carotene, zeaxanthin, and lutein, which are well known to have a strong antioxidant property, showed TEAC values at a range of 1400–2900 μM TE at 100 μM [36]. Therefore, FCX has a higher TAC compared to these carotenoids. The antioxidant properties of FCX are partly attributed to its unique structure that contains an allenic bond and oxygenic functional groups, including epoxy, hydroxyl, carbonyl, and carboxyl groups, in the polyene hydrocarbon chain. The presence of an allenic bond in FCX confers a potent free radical scavenging activity [11]. Also, the induction of antioxidant genes by FCX likely contributes to the inhibition of cellular ROS accumulation in macrophages. In the present study, we found that FCX significantly attenuated the decreased expression of antioxidant enzymes, such as *Sod1*, *Gpx1*, *Gpx4*, and *Cat*, by LPS. SOD1 (ZnSOD) and SOD2 (Mn-SOD) catalyze the dismutation of two O_2- to H_2O_2, which is then detoxificated into water and oxygen by catalase and GPxs [37]. Consistent with our findings, strong antioxidant effects of FCX have been well supported in vitro and in vivo studies. FCX significantly increased the protein levels of antioxidant enzymes, such as SOD2, GPx, and catalase, during differentiation of 3T3-L1 adipocytes [38]. Also, Ha et al. [31] showed that activities of antioxidant enzymes, such as catalase and GPx, were significantly increased in the liver of rats fed a high-fat diet containing FCX-rich powder. Thus, our results indicate that FCX-induced expression of antioxidant enzymes coupled with its potent ROS scavenging activity is a major contributor to the antioxidant effect of FCX.

Without oxidative stress, NRF2 is normally sequestered in the cytoplasm bound with its inhibitor, Keap1 [26]. ROS dissociate NRF2 from Keap1, so that NRF2 can enter the nucleus to bind an antioxidant response element (ARE) of its target genes, such as *Hmox1* [26]. We noticed in the present study that LPS markedly increased nuclear levels of NRF2, although it decreased *Nfe2l2* mRNA expression. Interestingly, the LPS-stimulated NRF2 nuclear translocation was further increased by FCX. It is possible that NRF2 nuclear translocation by LPS and FCX may be mediated by different mechanisms. The nuclear entry of NRF2 is likely elevated to counteract increased ROS production by LPS in macrophages. Interestingly, cellular ROS accumulation was markedly diminished by FCX concomitantly with marked increases in nuclear translocation of NRF2 and mRNA levels of *Nfe2l2* target genes, such as *Hmox1* and *Sod1*. The results suggest that FCX may directly target the activation of the NRF2 pathway to protect against oxidative stress by increasing antioxidant gene expression. For instance, HO-1

is known to regulate cellular redox homeostasis by inhibiting ROS generation, which can protect against oxidative stress and inflammation in bovine aortic endothelial cells [39]. Agmatine, an endogenous metabolite of L-arginine, increased LPS-induced *Hmox1* expression and nuclear NRF2 protein level, decreasing ROS production in macrophages [6]. Thus, FCX-induced nuclear translocation of NRF2 is likely contributed to the reduction of ROS accumulation by FCX.

Studies have shown that the nuclear translocation of NRF2 requires the activation of upstream kinases, such as PI3K and MAPKs, which can regulate ARE-mediated gene expression through the NRF2-dependent mechanism. The activation of MAPK signaling pathway induced ARE reporter gene expression, which was mediated via NRF2 in HepG2 cells [40]. Anthocyanins extracted from Korean black beans reduced the amyloid beta oligomer-induced oxidative stress and prevented neurodegeneration via the PI3K/AKT/NRF2-dependent pathway in mouse hippocampal HT22 cells [41]. Also, luteolin ameliorated $HgCl_2$-induced cardiac apoptosis and oxidative stress by activating PI3K/AKT/NRF2 signaling pathway in rats [42]. Luteolin-7-O-glucoside reduced t-BHP induced ROS generation, while it increased HO-1 protein expression and nuclear translocation of NRF2, which was mediated through the MAPK signaling pathway in RAW 264.7 cells [26]. We employed inhibitors of the kinases to explore their possible roles in the FCX-induced NRF2 nuclear translocation. We found that LY294002, an inhibitor of PI3K, but not MAPK inhibitors, attenuated an increase in the nuclear translocation of NRF2 by LPS and FCX with the latter being greater. Interestingly, FCX markedly increased AKT phosphorylation in LPS-induced macrophages. Thus, FCX-induced nuclear translocation of NRF2 is likely through the PI3K and AKT pathway, which are upstream kinases of NRF2. Studies have demonstrated that other bioactive food compounds also activate the NRF2 pathway via PI3K/AKT activation. Resveratrol increased *Hmox1* expression by activating NRF2 in PC12 cells, a rat pheochromocytoma cell line, which was attenuated by LY294002 [43]. Carnosol, a diterpene derived from the herb rosemary, increased *Hmox1* expression and nuclear protein levels of NRF2, but LY29400 inhibited carnosol-induced activation of AKT and NRF2 in PC12 cells [44]. Our results suggest that FCX activates the PI3K/AKT pathway for the induction of NRF2 nuclear translocation to exert its antioxidant effects. Focal adhesion kinase (FAK) is known to activate the PI3K/AKT and regulate adhesion signaling and motility in various cell types, including macrophages [45, 46]. Therefore, future research is warranted to identify upstream elements of the PI3K/AKT, such as FAK, which may be regulated by FCX to exert its antioxidant function.

The activation of NRF2 and the resulting induction of antioxidant genes are known to regulate inflammatory responses. LPS injection to *Nfe2l2*-deficient mice induced greater expression of pro-inflammatory cytokines, such as *Tnf* and *Il1α*, in the lung compared with wild-type mice [47]. However, it is noteworthy that we previously reported that the anti-inflammatory effect of berry anthocyanin fraction was independent of NRF2 in LPS-induced macrophages [21]. In LPS-stimulated bone marrow-derived macrophages (BMDM) from wild-type mice, berry anthocyanin fraction significantly decreased *Il1β* mRNA levels with a concomitant decrease in cellular ROS levels. However, in LPS-stimulated BMDM from *Nfe2l2*-deficient mice, the anthocyanin fractions significantly lowered *Il1β* mRNA without reducing cellular ROS levels [21]. Also, we demonstrated that astaxanthin significantly increased *Il1β* mRNA in LPS-stimulated BMDM from *Nfe2l2*-deficient mice [7]. In the current study, FCX significantly attenuated LPS-induced expression and secretion of pro-inflammatory cytokines, which may be attributed to its role in the activation of the NRF2 pathway to produce antioxidant enzymes. Also, LPS increased the nuclear translocation of NRF2, which was further increased by FCX. Thus, it is possible that FCX might suppress the inflammatory response independent of NRF2 in macrophage. The understanding of molecular bases for the anti-inflammatory effect of FCX independent of its antioxidant properties is warranted.

FCX can be biotransformed to FCN in the gastrointestinal tract by digestive enzymes, such as lipase and cholesterol esterase, and FCN is further converted into ACXA by short-chain dehydrogenase/reductase in the liver [19, 29]. Hashimoto et al. [29] demonstrated that dietary FCX preferentially accumulates as ACXA in the adipose tissue, while FCN is a primary FCX metabolite in other tissues of mice, including liver, lung, kidney, heart, and spleen. FCN and ACXA are considered to be active metabolites of FCX with physiological functions in the body [48]. FCN and ACXA are contributed to the bioactivities of FCX and play an important role in health benefits of FCX [49]. Thus, we included in our investigation FCN and ACXA to gain better mechanistic insight into the anti-inflammatory and antioxidant effects of FCX. FCN and ACXA suppressed LPS-induced pro-inflammatory gene expression and intercellular ROS accumulation in RAW 264.7 macrophages. The results suggest that FCN and ACXA are likely to contribute to the inhibitory effects of FCX on oxidative stress and inflammation in macrophages. Future study is warranted to determine the underlying molecular mechanism for antioxidant and anti-inflammatory effects of FCN and ACXA in macrophages in vivo and in vitro.

In summary, the present study shows that FCX exerts anti-inflammatory and antioxidant effects by activating

the NRF2 pathway in macrophages exposed to LPS. The increase in NRF2 nuclear translocation is mediated, at least in part, through the activation of PI3K/AKT pathway. Furthermore, we also show that two major FCX metabolites, i.e., FCN and ACXA, exert antioxidant and ant-inflammatory effects. This study shows for the first time the effect of FCX and its metabolites on the modulation of the PI3K/AKT/NRF2 to exert antioxidant and anti-inflammatory properties in macrophages. FCX and its metabolites have a high potential as anti-inflammatory and antioxidant agents to prevent and treat diseases inflicted by inflammation and oxidative stress.

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Author contributions MBK: conducted experiments, analyzed data, and wrote the manuscript. HK, YL, and YKP: performed experiments and contributed to manuscript preparation. JYL: designed the study, directed the study, interpreted data, and contributed to manuscript preparation.

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