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ORIGINAL CONTRIBUTION

Unsaturated fatty acids repress the expression of adipocyte fatty acid binding protein via the modulation of histone deacetylation in RAW 264.7 macrophages

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Abstract
Background Adipocyte fatty acid binding protein (A-FABP) present in macrophages has been implicated in the integration of lipid metabolism and inflammatory response, contributing to development of insulin resistance and atherosclerosis.
Aim of the study This study was conducted to test the hypothesis that the role of fatty acids in the inflammatory pathways is mediated through the modulation of A-FABP expression in macrophages.
Methods Murine RAW 264.7 macrophages were treated with inflammatory insults and fatty acids for quantitative real-time PCR and Western blot analysis. The cells were treated with trichostatin A (TSA), a histone deacetylase inhibitor, for elucidating mechanisms for the regulation of A-FABP expression by fatty acids. RNA interference (RNAi) to knock down A-FABP was utilized to assess its role in inflammatory gene expression.
Results When RAW 264.7 were incubated with lipopolysaccharides (LPS; 100 ng/ml) or 2.5 ng/ml of tumor necrosis factor α for 18 h, A-FABP mRNA and protein levels were drastically increased. Unsaturated fatty acids (100 μmol/l in complexed with BSA) such as palmitoleic acid, oleic acid, linoleic acid, linolenic acid, and eicosapentaenoic acid, significantly repressed the basal as well as LPS-induced A-FABP expression, whereas palmitic acid did not elicit the same effect. TSA increased A-FABP mRNA levels and abolished the repressive effect of linoleic acid on A-FABP expression in unstimulated and LPS-stimulated macrophages. Depletion of A-FABP expression by 70–80% using RNAi markedly decreased cyclooxygenase 2 mRNA abundance and potentiated the repression by linoleic acid.
Conclusion Unsaturated fatty acids inhibited the basal as well as LPS-induced A-FABP expression. The mechanism may involve histone deacetylation and anti-inflammatory effect of unsaturated fatty acids may be at least in part attributed to their repression of A-FABP expression in RAW 264.7 macrophages.

Keywords Adipocyte fatty acid binding protein · Fatty acids · RAW 264.7 macrophages · Histone deacetylase · Cyclooxygenase 2

Abbreviations

A-FABP	Adipocyte fatty acid binding protein
AP-1	Activator protein 1
BSA	Bovine serum albumin
COX-2	Cyclooxygenase 2
CVD	Cardiovascular disease
FBS	Fetal bovine serum
HDACi	Histone deacetylase inhibitor
IKK	I kappa B kinase
IL-1β	Interleukin-1β
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein 1

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NF-κB	Nuclear factor kappa B
PPARγ	Peroxisome proliferator-activated receptor γ
qPCR	Quantitative real-time PCR
siRNA	Small interfering RNA
TNFα	Tumor necrosis factor α
TSA	Trichostatin A

Introduction

Adipocyte fatty acid binding protein (A-FABP) or fatty acid binding protein 4 is a cytoplasmic lipid chaperon that binds with high affinity to hydrophobic ligands such as fatty acids and their derivatives [1]. A-FABP is abundantly expressed in adipocytes and to a lesser extent in macrophages [2–4]. It has been used as a model for differentiation-dependent gene expression in 3T3-adipocytes as its expression is transcriptionally activated during differentiation [5, 6].

A-FABP has been suggested to play an important role in the development of insulin resistance and atherosclerosis. Mice with A-FABP deficiency showed protection against the development of insulin resistance compared with wild-type animals [7–9]. Total and macrophage-specific A-FABP knockout mice exhibited a marked reduction in atherosclerotic lesion formation in apolipoprotein E knockout (*apoE*$^{-/-}$) mice [3, 10–12]. Additionally, administration of an A-FABP inhibitor in *ob/ob* and *apoE*$^{-/-}$ mice inhibited the development of type 2 diabetes and atherosclerosis, respectively [13]. In humans, a functional genetic variant of A-FABP gene, resulting in reduced A-FABP expression, showed a low risk of type 2 diabetes and cardiovascular disease (CVD) [14]. Increased plasma A-FABP concentrations in obese subjects correlate with insulin resistance, dyslipidemia, and metabolic syndrome [15–20]. The function of circulating A-FABP is unclear, but it may link obesity, inflammation, and metabolic syndrome [4].

The health benefits from absence or reduction of A-FABP are linked to inhibited production of inflammatory mediators in macrophages. In macrophage cell line, absence or chemical inhibition of A-FABP markedly diminished expression of pro-inflammatory cytokines and chemokines, including monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), and IL-6 [10, 13]. These effects in macrophages are primarily attributed to the role of A-FABP in peroxisome proliferator-activated receptor γ (PPARγ) and nuclear factor kappa B (NF-κB) pathways [21, 22].

Fat overload and increased circulating free fatty acid concentrations in obesity are a key metabolic component to trigger the activation of inflammatory pathways. In general, saturated fatty acids are believed to be pro-inflammatory, whereas unsaturated fatty acids, n-3 polyunsaturated fatty acids in particular, have an anti-inflammatory property. In THP-1 cells, long-chain unsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid significantly lowered inflammatory gene expression compared with saturated fatty acids [23]. Repression of pro-inflammatory gene expression by γ-linolenic acid is shown to be mediated through the inhibition of NF-κB and activator protein 1 (AP-1) [24]. However, our understanding on molecular mechanisms and identification of mediators to deliver the effect of fatty acids on the integration of metabolic and inflammatory pathways is surprisingly limited. Given the role of A-FABP in cellular trafficking of fatty acid and their metabolites as well as inflammatory pathways, A-FABP could play a pivotal role in delivering various metabolic effects of fatty acids and could link metabolic signatures to inflammatory response in macrophages. This study was undertaken to evaluate the effect of inflammatory stimuli and fatty acids on the expression of A-FABP and to elucidate molecular mechanisms underlying the effect.

Methods and materials

Cell culture and fatty acid preparation

Murine RAW 264.7 macrophages (ATCC) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 1× vitamins, and 2 mmol/l L-glutamine in a humidified chamber at 37 °C with 5% CO_2. All cell culture supplies were purchased from MediaTech.

RAW 264.7 macrophages were incubated with 100 ng/l lipopolysaccharides (LPS; Sigma-Aldrich) or 2.5 ng/ml of tumor necrosis factor α (TNFα; eBioscience) for 18 h. For incubation with fatty acids, 2 mmol/l of fatty acid-poor and endotoxin-free bovine serum albumin (BSA; Calbiochem) was prepared in PBS. Sodium salts of fatty acids (Nu-Chek) were dissolved in the BSA stock solution to a concentration of 5 mmol/l. After purged with N_2, the solution was sonicated in a water bath until it became clear to form BSA/fatty acid complex (molar ratio to BSA of 1:2.5). The complex was sterilized through a Millex®-GV 0.22 µM filter unit (Millipore) and diluted with cell medium to reach a final concentration of 100 µmol/l. Fatty acids prepared include palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), and eicosapentaenoic acid (20:5). RAW 264.7 macrophages were incubated with BSA only

(control) or 100 μmol/l of a fatty acid for 6 h followed by additional 18 h with 100 ng/l of LPS. For experiments with trichostatin A (TSA; Sigma–Aldrich), a pan-histone deacetylase inhibitor (HDACi), RAW 264.7 macrophages were treated with 100 μmol/l fatty acids in the presence or absence of 500 nmol/l of TSA in DMSO for 6 h followed by incubation with 100 ng/ml LPS for additional 14 h.

Small interfering RNA (siRNA) transfection

RAW 264.7 macrophages were transfected with Silencer® Negative Control scrambled siRNA (Ambion) or siGENOME® SMART pool A-FABP siRNA (Dharmacon) to knockdown A-FABP expression using DharmaFECT®1 transfection reagent (Dharmacon) according to the manufacture's protocol. In brief, transfection reagent was prepared by dilution using cell medium void of antibiotics and FBS with a factor of 40. siRNA in RNase-free sterile water (2 μmol/l) was mixed with equal volume of cell medium void of antibiotics and FBS. Subsequently, the media containing transfection reagent and siRNA were combined and incubated for 20 min at room temperature and subsequently added to cells. Twenty-four hour after transfection, the cells were washed twice with PBS and incubated with 100 μmol/l fatty acids for 6 h followed by 100 ng/ml LPS for 18 h.

Total RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and qPCR was conducted as previously described [25, 26]. Primers were designed using Primer Express software (Applied Biosystems) according to GenBank database: A-FABP, forward (5′-CACCATCCG GTCAGAGAGTACTT-3′), reverse (5′-CGGTGATTTCA TCGAATTCCA-3′); Mal1, forward (5′-CGACAGCTGA TGGCAGAAAA-3′), reverse (5′-CAGGGCACCGTCTTG GAA-3′); cyclooxygenase 2 (COX-2), forward (5′-AAAG GTTCTTCTACGGAGAGAGTTCA-3′), reverse (5′-TGG GCAAAGAATGCAAACATC-3′); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward (5′-GTGGTCT CCTCTGACTTCAACA-3′), reverse (5′-GTTGCTGTAGC CAAATTCGTTGT-3′).

Western blot analysis

Cell lysate was prepared, and Western blot analysis was performed as previously described [25, 26]. A-FABP/FABP4 antibody was purchased from Santa-Cruz. Monoclonal antibody against β-actin was obtained from Sigma for a loading control to normalize the data.

Statistical analysis

ANOVA and Newman–Keuls multiple comparison with Welch's correction for unequal variance when appropriate were used to identify statistically significant differences of treatments with $P < 0.05$ considered significant by GraphPad InStat 5 (GraphPad Software, Inc.). Data are expressed as mean ± SEM.

Results

Induction of A-FABP expression by inflammatory agents

To evaluate the effect of inflammatory agents on macrophage A-FABP expression, RAW 264.7 macrophages were treated with either 100 ng/ml LPS or 2.5 ng TNFα for qPCR analysis. A-FABP mRNA levels were significantly increased by both inflammatory agents by ~2.5- to 8-fold relative to the control (Fig. 1).

Inhibition of A-FABP expression by unsaturated fatty acids

Modulation of A-FABP expression by various fatty acids in unstimulated and LPS-stimulated RAW 264.7 macrophages was determined. The cells were incubated with fatty acids, including 16:0, 16:1, 18:1, 18:2, 18:3, 20:4, and 20:5, in the absence or presence of LPS. All of the unsaturated fatty acids tested significantly lowered A-FABP mRNA abundance in both unstimulated and LPS-stimulated macrophages, whereas 16:0 did not induce the reduction compared with control (Fig. 2a). Western blot analysis confirmed reduced A-FABP protein levels by unsaturated fatty acids with 20:5 having the greatest effect (Fig. 2b). Interestingly, despite no difference in mRNA

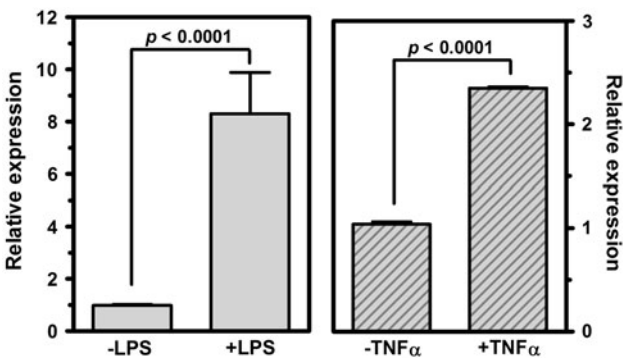

Fig. 1 Induction of A-FABP expression by inflammatory agents in RAW 264.7 macrophages. Cells were treated with 100 ng/l LPS or 2.5 ng/l TNFα for 18 h. A-FABP mRNA expression was determined by qPCR. $n = 9$, Mean ± SEM

abundance was observed between the control and 16:0-treated cells, A-FABP protein levels were markedly higher in 16:0 than control in the presence of LPS.

Abrogation of unsaturated fatty acid-mediated repression of A-FABP expression by TSA

We explored whether altered histone acetylation state is involved in the regulation of macrophage A-FABP expression in response to LPS and fatty acids in RAW 264.7 macrophages. Cells were treated with TSA to block HDAC activity in the absence or presence of fatty acids and LPS. In unstimulated cells, LPS significantly increased A-FABP mRNA levels and 18:2 markedly reduced A-FABP expression in both stimulated and unstimulated macrophages, which is consistent with our observations shown in Figs. 1 and 2 (Table 1). TSA treatment significantly elevated A-FABP mRNA levels, but the repressive effect of 18:2 was completely abolished both in the absence and the presence of LPS.

Role of A-FABP in the regulation of COX-2 expression by fatty acids in RAW 264.7 macrophages

To further assess the role of A-FABP in inflammatory property of fatty acids in RAW 264.7 macrophages, A-FABP was knocked down by siRNA and cells were treated with fatty acids and LPS. We achieved A-FABP knockdown by ~75–80%, and depletion of A-FABP was not compensated by Mal1, another major fatty acid binding protein in macrophages (Fig. 3a). Interestingly, basal expression of COX-2 was increased in the fatty acid-treated cells compared with control when A-FABP was depleted compared with scrambled control (Fig. 3b). In LPS-activated cells, 18:2 induced significantly lower COX-2 mRNA abundance than control and 16:0. Depletion of A-FABP markedly reduced COX-2 mRNA levels in all treatments, and the repressive effect of 18:2 on COX-2 expression was further potentiated in LPS-treated macrophages. In contrast, when cells were stimulated by LPS, A-FABP knockdown had a minimal effect on IL-1β, TNFα, and IL-6 mRNA levels (data not shown).

Table 1 Abrogation of unsaturated fatty acid-mediated repression of A-FABP expression by TSA in RAW 264.7 macrophages

TSA	LPS	Control	16:0	18:2
−	−	1.00 ± 0.01^{b}	2.47 ± 0.31^{c}	0.31 ± 0.02^{a}
−	+	3.30 ± 0.79^{b}	4.02 ± 0.33^{b}	0.63 ± 0.13^{a}
+	−	3.30 ± 0.31^{a}	5.16 ± 0.59^{b}	2.97 ± 0.36^{a}
+	+	6.35 ± 1.10	6.73 ± 0.15	6.07 ± 1.69

Mean ± SEM. $n = 9$. Data with a different superscript in the same row are significantly different ($P < 0.05$)

Discussion

Studies in animals and humans have revealed a key role of A-FABP in accelerating the development of type 2 diabetes and CVD, which is attributed, at least in part, to its role in

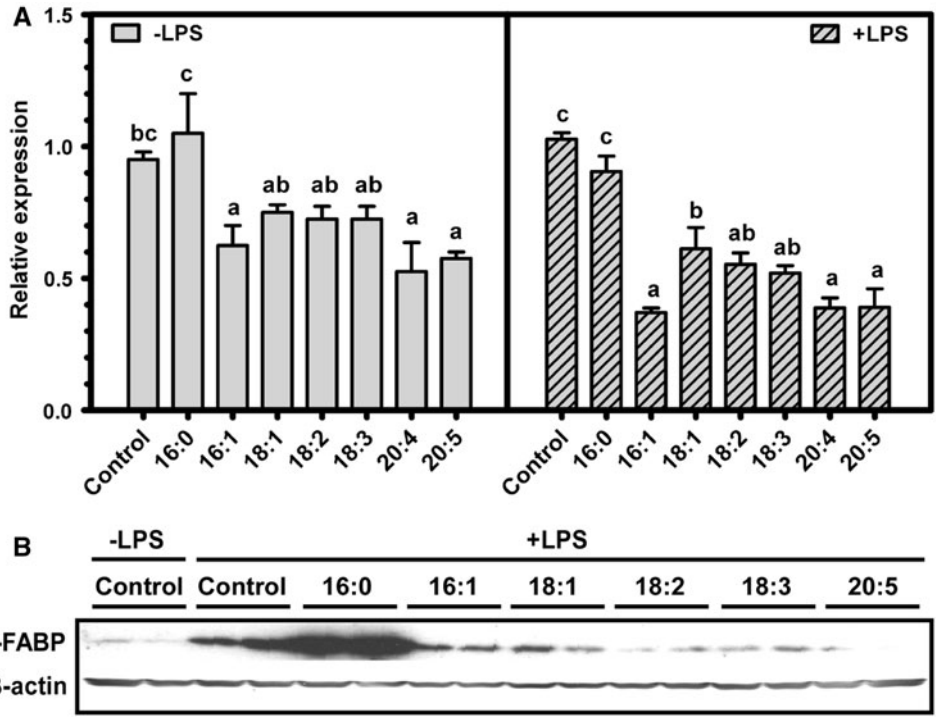

Fig. 2 Inhibition of A-FABP expression by unsaturated fatty acids in RAW 264.7 macrophages. Cells were treated with 100 μmol/L fatty acid complexed with BSA for 12 h and subsequently with 100 ng/ml LPS for additional 18 h. A, A-FABP mRNA expression levels were measured by qPCR. $n = 3$–4, Mean ± SEM, $P < 0.05$. B, after treatment with fatty acid and LPS as described above, cell lysate was prepared for Western blot analysis for A-FABP. β-actin was used as a loading control, and a representative blot of two separate experiments is shown

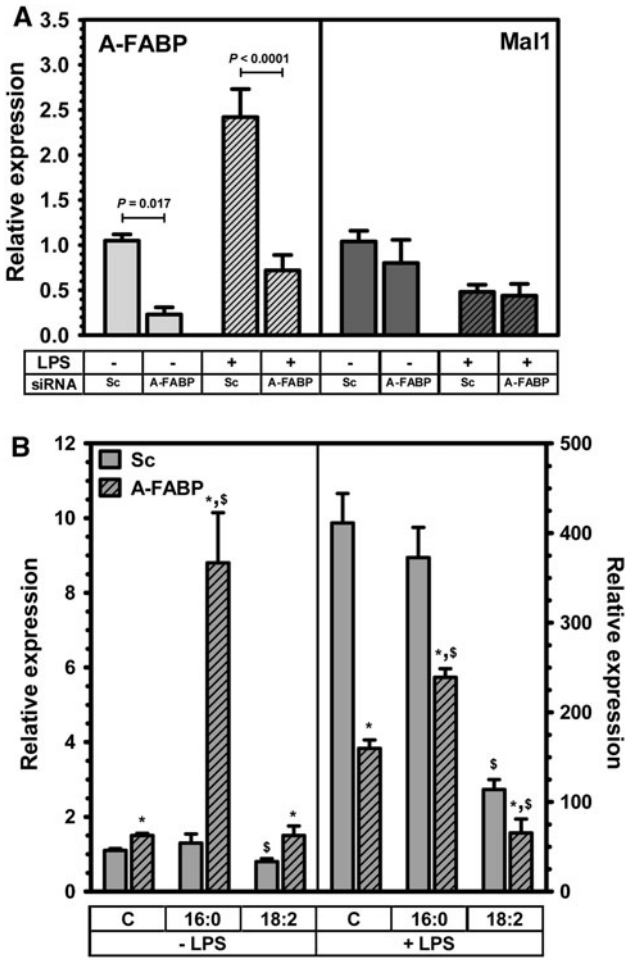

Fig. 3 Role of A-FABP in the regulation of COX-2 expression by fatty acids in RAW 264.7 macrophages. Cells were pre-treated with 100 μmol/l fatty acid and 500 μmol/l TSA for 6 h, after which they were incubated with 100 ng/ml of LPS for additional 18 h in the presence of fatty acid and TSA. mRNA expression for A-FABP and Mal1 (**a**) and COX-2 (**b**) was measured by qPCR. $n = 8$-9, Mean ± SEM. *, $P < 0.05$ compared with a scrambled control in the same fatty acid and LPS treatment. $, $P < 0.05$ compared with BSA control in the same LPS and siRNA treatment

activating inflammatory signaling pathways in macrophages. Despite a potential role of A-FABP in the integration of cell's metabolic signatures with inflammatory responses, detailed regulatory mechanisms for A-FABP expression have been understudied in macrophages. We found that A-FABP expression in macrophages is repressed by unsaturated fatty acids and acetylation of histones is likely to play a role in this repression. In addition, A-FABP may contribute to the induction of COX-2 expression by inflammatory insults in macrophages.

In the present study, both LPS and TNFα significantly increased A-FABP expression in RAW 264.7 macrophages. The elevation of A-FABP expression by inflammatory stimuli is consistent with findings in literature [10, 27]. Mechanisms underlying this induction, however, are elusive. Although cell surface receptors for LPS and TNFα are different, downstream mediators that can transduce their signals can converge onto similar pathways, including I kappa B kinases (IKK)/NF-κB and mitogen-activated protein kinases (MAPK)/AP-1 pathways. Our observation that both LPS and TNFα induced A-FABP mRNA expression indicates that common elements downstream of LPS and TNFα pathways are likely involved in the induction. Although PPARγ is a well-known transcriptional regulator of A-FABP in adipocytes [28], we speculate that the induction of A-FABP expression by inflammatory stimuli in macrophages may not be PPARγ dependent. Several studies have shown that phosphorylation of PPARγ by inflammatory stimuli-activated MAPK inhibits transcriptional activity of PPARγ [29–32]. If PPARγ is a major regulatory factor for A-FABP expression in macrophages, we would expect down-regulation, not up-regulation, of A-FABP expression in LPS and TNFα-activated macrophages. AP-1 and CCAAT enhancer binding protein are also shown to regulate A-FABP expression in adipocytes and pre-adipocytes [33–36]. Recently, Hui et al. [37] reported that in macrophages, increased A-FABP expression via activation of c-Jun N-terminal kinase/c-Jun pathway. Considering our observation that TSA treatment increased basal as well as LPS-induced A-FABP mRNA levels, whether AP-1 regulates A-FABP expression by utilizing histone deacetylase in macrophages is worthwhile to investigate.

Basal as well as LPS-induced A-FABP expression in RAW 264.7 macrophage was repressed by unsaturated fatty acids but not by saturated fatty acids in the present study. As TSA treatment completely abolished the repressive effect of unsaturated fatty acids in basal and LPS-induced A-FABP mRNA levels, we speculate that unsaturated fatty acids may inhibit A-FABP expression by modulating histone deacetylase in the promoter of A-FABP. As only a specific small number of genes (1–7%) are shown to be altered by HDACi [38–41], the TSA effect on A-FABP is not likely to be general but rather specific to the gene. Transcriptional factors namely AP-1, NF-κB, and liver X receptor α [42–46] have shown to exert active transrepression of gene expression through association with a co-repressor complex containing a HDAC when they are not activated by their agonists. It is tempting that unsaturated fatty acids may increase HDAC activity in a co-repressor complex present in the promoter of A-FABP. Further study is necessary to address this possibility.

Of interest in this study is that potential post-transcriptional regulation of A-FABP by fatty acids was observed in addition to the transcriptional regulation described above. When macrophages were treated with 16:0, there was no significant difference in A-FABP mRNA levels between control and the fatty acid-treated cells. A-FABP protein levels, however, were markedly increased in 16:0-treated

group. Furthermore, 20:5 drastically lowered A-FABP protein compared with other unsaturated fatty acids, despite mRNA levels were not significantly altered. It needs to be determined whether fatty acids modulate translation efficiency and/or degradation of A-FABP in macrophages.

COX-2 is one of two major COX isoforms that can convert arachidonic acid to prostanoids [47]. Whereas COX-1 is known to be constitutively expressed, COX-2 is inducible by various inflammatory stimuli such as LPS and has been linked to atherosclerosis development [48, 49]. Based on initial observations of inflammatory properties of COX-2, developing COX-2-specific inhibitors was considered as a promising drug therapy for inflammatory diseases, including atherosclerosis. However, COX-2-specific inhibitors in human clinical trials increased heart attacks and strokes [50, 51]. This outcome may result from disruption of balance between pro- and anti-inflammatory prostanoids. Therefore, it would be beneficial to find a means to gently alter COX-2 function. In the present study, 18:2 significantly inhibited the expression of TNFα, IL-6, IL-1β, and COX-2 in LPS-stimulated macrophages. However, it was only COX-2 whose expression was further diminished by A-FABP depletion. Mechanisms by which A-FABP affects COX-2 expression in macrophages need further investigation.

In summary, we have shown a significant up-regulation of A-FABP by inflammatory insults and that unsaturated fatty acid repress both basal and induced A-FABP expression in RAW 264.7 macrophages. The repression of A-FABP expression by unsaturated fatty acids was completely abolished by an HDACi, suggesting that alterations in histone acetylation state in A-FABP promoter may be an important mechanism for the regulation of A-FABP transcription by fatty acids in macrophages. Lastly, we also show that a relationship between COX-2 and A-FABP could be a mechanism for the anti-inflammatory effects of unsaturated fatty acids in macrophages. Due to the high frequency of obesity in the United States, deciphering the mechanism for the anti-inflammatory properties of unsaturated fatty acid will allow for more appropriate and precise dietary interventions to prevent chronic metabolic and inflammatory diseases.

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Conflicts of interest All authors read and approved the final manuscript and have no conflicts of interest.

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