



Exogenous FABP4 interferes with differentiation, promotes lipolysis and inflammation in adipocytes

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Abstract

Purpose Fatty acid binding protein 4 (FABP4) has been demonstrated to be secreted from adipocytes in an unconventional pathway associated with lipolysis. Circulating FABP4 is elevated in metabolic disorders and has been shown to affect various peripheral cells such as pancreatic β -cells, hepatocytes and macrophages, but its effects on adipocytes remains unclear. The aim of this study was to investigate the effects of exogenous FABP4 (eFABP4) on adipocyte differentiation and function.

Methods 3T3-L1 pre-adipocytes or mature adipocytes were treated with recombinant FABP4 in the absence or presence of FABP4 inhibitor I-9/p38 MAPK inhibitor SB203580; Meanwhile male C57BL/6J mice were subcutaneously injected twice a day with recombinant FABP4 (0.35 mg/kg) with or without I-9 (50 mg/kg) for 2 weeks. The effects of eFABP4 on differentiation, lipolysis and inflammation were determined by triglyceride measurement or lipolysis assay, western blotting, or RT-qPCR analysis.

Results eFABP4 treatment significantly reduced intracellular triglyceride content and decreased expression of adipogenic markers peroxisome proliferator-activated receptor gamma (PPAR γ), CCAAT/enhancer binding protein alpha (C/EBP α), intracellular FABP4, and adiponectin in 3T3-L1 cells. Besides, eFABP4 promoted lipolysis and inflammation in differentiated 3T3-L1 adipocytes as well as in adipose tissue of eFABP4-treated C57BL/6J mice, with elevated gene expression of monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)- α , and elevated protein expression of adipose triglyceride lipase (ATGL), phosphorylation of hormone-sensitive lipase (HSL) (Ser-660), p38, and nuclear factor-kappa B (NF- κ B). The pro-inflammatory and pro-lipolytic effects of eFABP4 could be reversed by SB203580/I-9.

Conclusions These findings indicate that eFABP4 interferes with adipocyte differentiation, induces p38/HSL mediated lipolysis and p38/NF- κ B mediated inflammation in adipocytes in vitro and in vivo.

Keywords eFABP4; Adipocyte; Differentiation; Lipolysis; Inflammation

Introduction

As a major site for energy storage and lipid metabolism, adipose tissue plays an indispensable role in the homeostasis of body system [1, 2]. Dysfunction of adipocytes has wide-ranging effects, contributing to a variety of

metabolic disorders including obesity and diabetes [3]. Adipocytes maintain lipid homeostasis by regulating adipogenesis and lipolysis [4], while impaired adipogenesis and increased free fatty acids (FFAs) by excess lipolysis subsequently result in ectopic lipid accumulation and lipotoxicity, a mechanism known in the development of type 2 diabetes [5, 6]. Adipocytes also act as endocrine cells responsible for expression and secretion of multiple adipokines, such as adiponectin, FABP4, monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, and tumor necrosis factor (TNF)- α [7, 8]. The profile of secreted adipokines becomes altered in pathological status including chronic low-grade inflammation, another key factor involved in the initiation and development of metabolic diseases [9, 10]. p38 MAPK has been reported to be involved in lipid metabolism and also participate in

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regulating adipocyte inflammation with subsequently activation of NF- κ B [11–13].

FABP4 is a 14.6 kDa protein highly expressed in adipocytes and also readily expressed in other cell types such as macrophages and endothelial cells, serving as a lipid chaperone that regulates transport, metabolism, and storage of lipids [14, 15]. FABP4 deficient mice are protected against insulin resistance and type 2 diabetes in the context of dietary or genetic obesity [16]. Decreased lipolysis is detected in adipocytes from FABP4 deficient mice, probably attributing to a direct interaction of FABP4 with hormone-sensitive lipase (HSL) [16–18]. FABP4-null pre-adipocytes exhibit a potentially enhanced peroxisome proliferator-activated receptor gamma (PPAR γ) expression and adipogenesis compared with wild-type cells [19]. Targeting FABP4 provides therapeutic possibilities with great potential for treating various metabolic disorders [20]. FABP4 is not only a cytoplasmic protein but also can be secreted from adipocytes in association with lipolysis via a nonclassical secretion pathway [21–23]. Serum FABP4 levels are markedly elevated in metabolic disorders such as obesity [24], insulin resistance [25], type 2 diabetes [26], atherosclerosis [27], and cardiovascular events [28]. Thus, it can be used for the clinical diagnosis of obesity-related metabolic diseases and cardiovascular diseases [26, 29]. Despite the robust associations, the mechanisms underlying how FABP4 contributes to disease pathogenesis remains enigmatic. Circulating FABP4 has been shown to act as a bioactive molecule that regulates the function of numerous cells or tissues, such as potentiating glucose-stimulated insulin secretion in pancreatic β cells [30], enhancing hepatic glucose production in vitro and in vivo [31], aggravating the endoplasmic reticulum stress in HepG2 cells [32], attenuating the insulin signaling pathway in C2C12 myotubes [33], inducing acute calcium-dependent myocardial contraction disorder and myocardial neutral lipid accumulation [34, 35], inhibiting the activation of endothelial nitric oxide synthase and impairing the insulin-dependent nitric oxide pathway in vascular endothelial cells [36, 37], and inducing the expression of inflammatory genes in combination with palmitic acid in macrophages [36]. However, the effects of eFABP4 on adipocytes remain unclear so far. Herein, we aimed to explore the potential effects of eFABP4 on adipocyte differentiation and function, and further investigated the possible signaling pathway involved.

Materials and methods

Reagents

Thiazolyl blue tetrazolium bromide (MTT), bovine insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were

obtained from Sigma-Aldrich. SB203580 was purchased from Selleck. FABP4 inhibitor I-9 was discovered by our laboratory and synthesized as previously described [38]. Oil red O was purchased from AMRESCO. Regents for cell culture were purchased from Thermo Fisher Scientific. Primary antibodies were obtained from Cell Signaling Technology. Secondary antibodies were obtained from Jackson Laboratory. Primers were synthesized by Shanghai Generay Biotech Co., Ltd. and the sequences were listed in Table 1.

Purification of recombinant FABP4

Recombinant mouse FABP4 with a 6 \times His tag was produced in *E. coli* using the pET28a vector and isolated by nickel-affinity chromatography as previously reported [39], and further purified with gel filtration chromatography (Superdex-200, GE Healthcare).

Cell culture

Mouse 3T3-L1 pre-adipocytes (CL-173) were purchased from American Type Culture Collection and maintained in DMEM containing 10% fetal bovine serum at 37 °C with 5% CO₂. The cells were differentiated into adipocytes as previously described [40].

Oil red O staining, TG measurement, and Lipolysis assay

Recombinant FABP4 with or without FABP4 inhibitor I-9 were added at the beginning of 3T3-L1 differentiation induction and incubated in the medium throughout the differentiation period (day 0–6). At day 6, oil red O staining

Table 1 Primer sequences

Gene name	Primer sequence
GAPDH	Forward: TGCACCACCAACTGCTTAGC
	Reverse: GGATGCAGGGATGATGTTCT
TNF- α	Forward: ATGGGAAGGGAATGAATCCACC
	Reverse: GTCCACATCCTGTAGGGCGTCT
MCP-1	Forward: CCACTCACCTGCTGCTACTCAT
	Reverse: TGGTGATCCTCTTGAGCTCTCC
IL-6	Forward: TCTGAAGGACTCTGGCTTTG
	Reverse: GATGGATGCTACCAAAGTGA
adiponectin	Forward: CAACCAACAGAATCATTATG
	Reverse: GGTAAGAGAAGTAGTAGAGT
PPAR γ	Forward: CAGGCTTCCACTATGGAGTTC
	Reverse: GGCAGTTAAGATCACACCTATCA
FABP4	Forward: GCGTAAATGGGGATTGGTC
	Reverse: CTCCTGTCGCTGCGGTGATT

or intracellular TG measurement were conducted respectively as previously reported [40]. Differentiated 3T3-L1 cells were pre-incubated with or without I-9/SB203580 for 2 h followed by recombinant FABP4 treatment for 48 h. Then the cells were assayed for lipolysis as previously described [40].

Animal treatment

Six-week-old male C57BL/6J mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and housed in a 12 h light-dark cycle with regular chow diet and free access to water. After adaptively fed for 1 week, mice were randomly divided into 3 groups ($n = 10$), subcutaneously injected twice a day with recombinant FABP4 (0.35 mg/kg, diluted in saline) with or without FABP4 inhibitor I-9 (50 mg/kg) by intragastric administration for 2 weeks. Vehicle group mice received an identical amount of 0.5% carboxymethyl cellulose and saline. On the 15th day, mice were sacrificed and epididymal adipose tissue was immediately removed, and frozen in liquid nitrogen.

All animal experiments were permitted by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica (accreditation number: 2018–10-WHY-16).

Immunoblot and gene expression analysis

Total protein or RNA were extracted from cells or epididymal adipose tissue and quantified by western blotting or real-time (RT)-PCR (RT-qPCR) as previously described [40].

Statistical analysis

Data were analyzed with GraphPad Prism software and expressed as mean \pm SEM of at least 3 independent experiments. Statistical analysis of different groups was performed by a two-tailed, unpaired Student's *t* test or one-way analysis of variance followed by a post hoc Tukey's test to make pairwise comparisons. A 95% confidence intervals was calculated and a value of $p < 0.05$ was considered significant.

Results

eFABP4 interferes with adipocyte differentiation

First, MTT assay revealed that eFABP4 had no significant effect on the viability of 3T3-L1 pre-adipocytes up to 8000 ng/mL (Data not shown). To investigate the effect of eFABP4 on adipocyte differentiation, intracellular

triglyceride measurement and oil red O staining were performed. The results revealed that eFABP4 dose-dependently decreased the intracellular triglyceride content and lipid-droplet formation of adipocytes compared with the control group (Fig. 1a, b). These findings suggested that eFABP4 exhibited an anti-adipogenic phenotype in the process of adipocyte differentiation.

To gain a better understanding of the molecular mechanisms underlying the anti-adipogenic effect of eFABP4, the expression levels of master adipogenic transcription factors PPAR γ , CCAAT/enhancer binding protein alpha (C/EBP α), as well as PPAR γ gene products FABP4 and adiponectin were analyzed. As shown in Fig. 1c, d, eFABP4 significantly decreased the protein expression of PPAR γ , C/EBP α , FABP4, and adiponectin in a dose-dependent manner. Following RT-qPCR results showed that eFABP4 also decreased PPAR γ , FABP4, and adiponectin expression on the mRNA level during adipocyte differentiation (Fig. 1e). The addition of FABP4 inhibitor I-9 reversed the inhibitory effect of eFABP4 on adipocyte differentiation, manifested as an increased TG content as well as increased expression of PPAR γ , C/EBP α , FABP4, and adiponectin (Fig. 1f).

eFABP4 promotes adipocyte lipolysis in vitro and in vivo

To investigate the effects of eFABP4 on mature adipocytes, fully differentiated 3T3-L1 adipocytes were incubated with eFABP4 (500 and/or 2000 ng/mL) in the presence or absence of 50 μ M I-9 for 48 h. As shown in Fig. 2a–c, eFABP4 dose-dependently induced both basal (15 and 26% increase) and forskolin-stimulated glycerol release (7 and 24% increase) from mature adipocytes, while I-9 inhibited eFABP4-induced glycerol release by 33% decrease compared with the eFABP4-treated group. To map signals beneath lipolysis, we examined the protein level of adipose triglyceride lipase (ATGL) as well as phosphorylation of HSL on key serine residues. ATGL content and reversible HSL phosphorylation are hallmarks of lipolysis regulation, and phosphorylation of HSL at serine-660 has been demonstrated to activate HSL activity [41]. As illustrated in Fig. 2d, e, ATGL protein level and phosphorylation of HSL at ser-660 were potently induced under eFABP4 treatment (54 and 37% increase, respectively), with I-9 reversed the induction by 53 and 45% decrease compared with the eFABP4-treated group.

If eFABP4 also functions to regulate adipocytes in vivo, there might be crucial physiological and pathophysiological implications of such activity and its aberrant regulation in immunometabolic disease. To verify the in vivo effect of eFABP4 on adipose tissue, we subcutaneously injected recombinant FABP4 into conscious C57BL/6J mice for

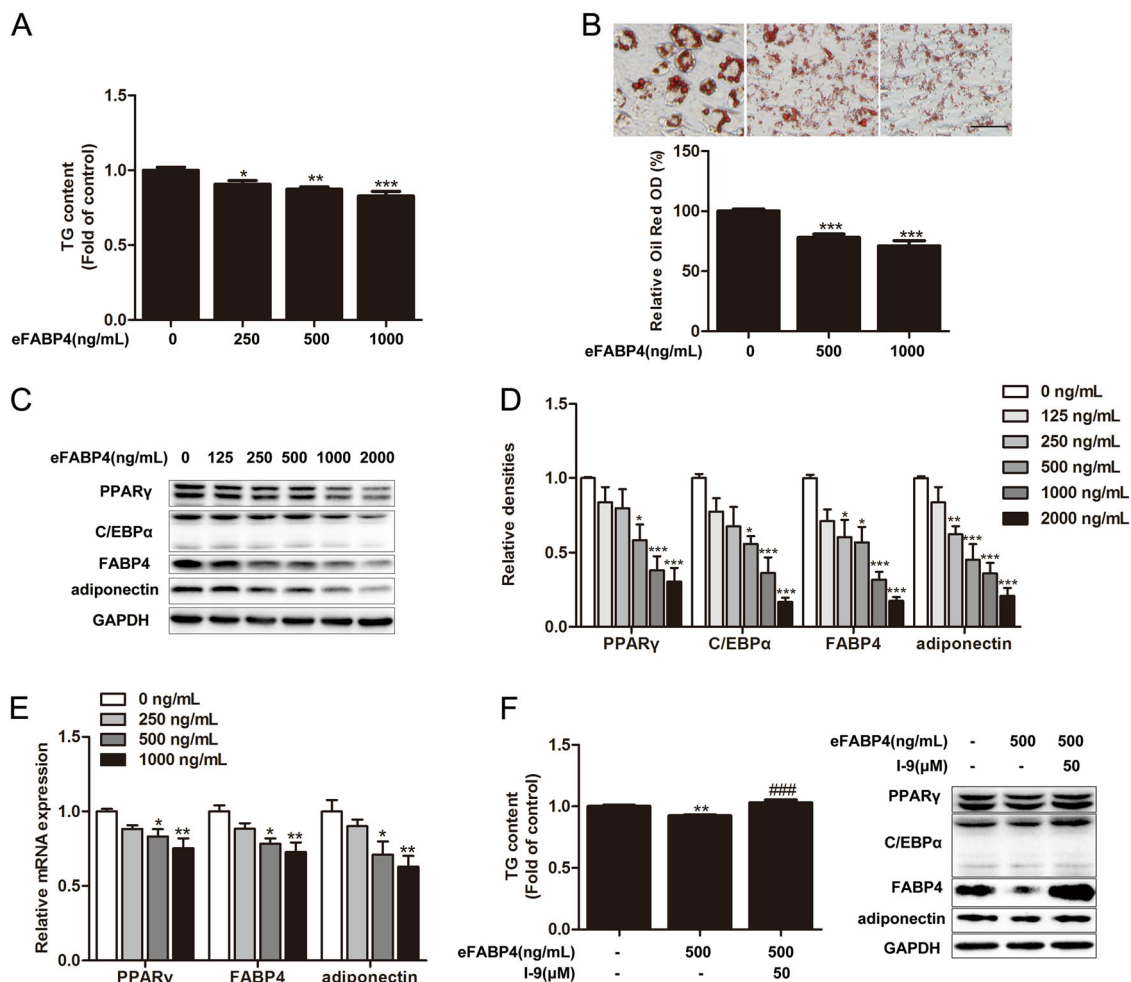


Fig. 1 Effects of eFABP4 on 3T3-L1 differentiation. TG determination (a, f) and oil red O staining (b) of 3T3-L1 cells after 6-day differentiation. c, d, f Protein levels of PPAR γ , C/EBP α , FABP4, and adiponectin. e Gene expression of PPAR γ , FABP4, and adiponectin.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group and #### $p < 0.0001$ vs. the eFABP4-treated group. Values are given as mean \pm S.E. M. ($n = 3$)

14 days, thus elevating serum FABP4 in otherwise metabolically normal mice to examine the effects of eFABP4 on adipose tissue. Similar to the in vitro results, the level of phosphorylated HSL (ser-660) was significantly upregulated, while ATGL showed an upward trend in epididymal adipose tissue of eFABP4-treated group (51 and 28% increase, respectively). FABP4 inhibitor I-9 reversed eFABP4-induced upregulation of ATGL and HSL phosphorylation in adipose tissue by 26 and 48% decrease compared with the eFABP4-treated group, respectively (Fig. 2f, g). Above all, we could draw a conclusion that eFABP4-induced adipocyte lipolysis.

eFABP4 induces adipocyte inflammation in vitro and in vivo

As eFABP4 has been reported to regulate the inflammatory response in macrophages and endothelial cells [37, 42], we

next detected the inflammatory reaction in adipocytes by eFABP4 treatment. As illustrated in Fig. 3a–d, eFABP4 treatment induced the phosphorylation of p38 (43% increase) and NF- κ B (21% increase) in differentiated 3T3-L1 adipocytes, but no significant change was observed on phosphorylation of JNK and ERK, while I-9 reversed the pro-inflammatory effect of eFABP4 by decreasing the phosphorylation of p38 and NF- κ B. Similarly, to verify the in vivo effect of eFABP4 on adipose tissue inflammation, we detected the inflammatory pathway of epididymal adipose tissue obtained from the aforementioned short term/high concentration of eFABP4 injection mice. As shown in Fig. 3e–g, eFABP4 treatment led to a 2.1-fold increase in both the phosphorylation of p38 and NF- κ B compared with the vehicle group, while no significant change was observed on phosphorylation of JNK. In addition, eFABP4 treatment upregulated the inflammatory genes MCP-1 (89% increase), TNF- α (76% increase), and IL-6 (61% increase) expression

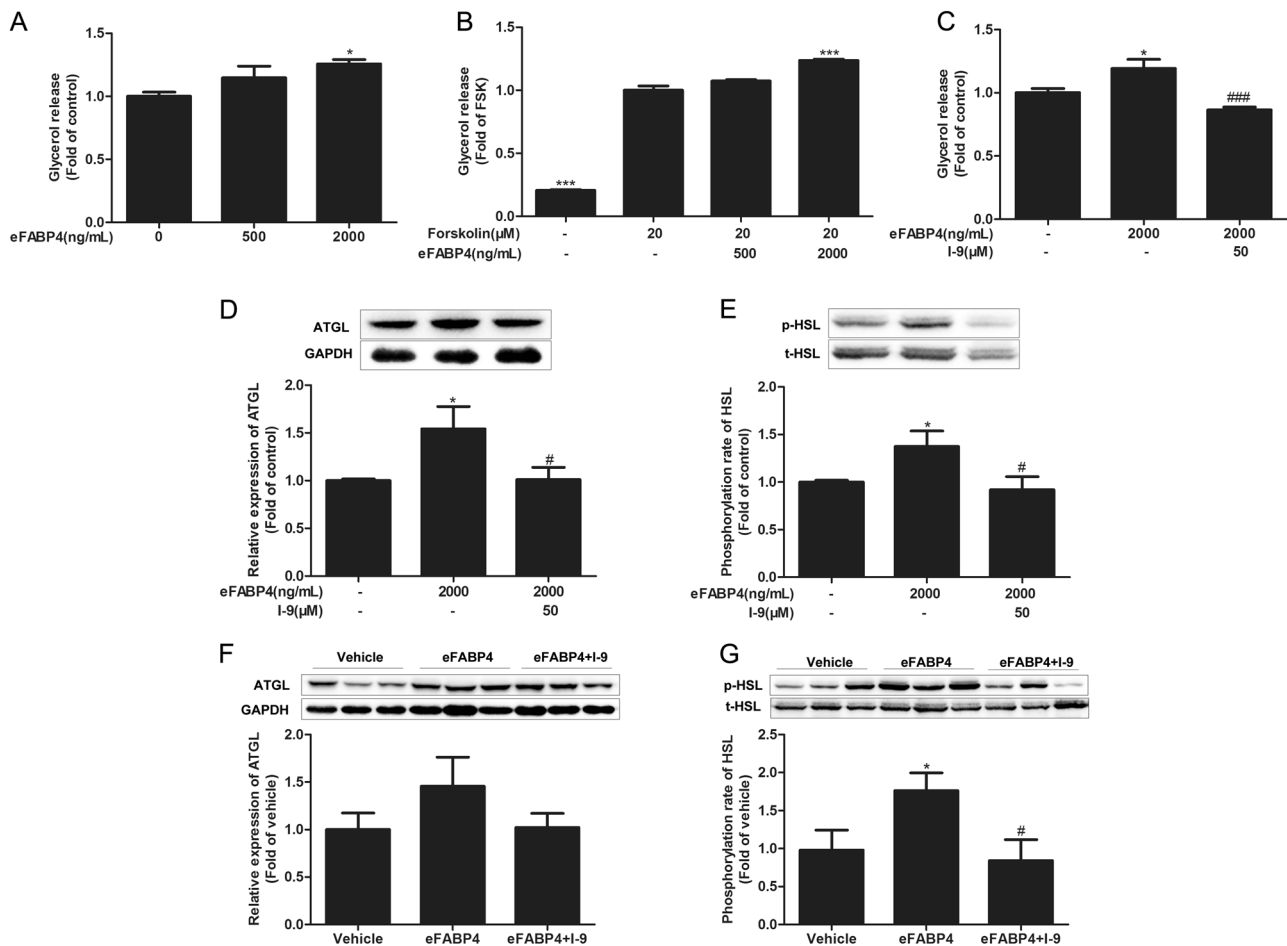


Fig. 2 eFABP4 treatment promoted lipolysis in both differentiated 3T3-L1 cells and epididymal adipose tissue of C57 mice. **a–c** Supernatant glycerol measurement. Western blot analysis of p-HSL (Ser-660), t-HSL, and ATGL of differentiated 3T3-L1 cells (**d, e**) and

epididymal adipose tissue (**f, g**). * $p < 0.05$ vs. control group; # $p < 0.05$, ### $p < 0.001$ vs. the eFABP4-treated group. Values are given as mean \pm S.E.M. ($n = 3$)

of adipose tissue, while I-9 downregulated these inflammatory genes (Fig. 3h). Moreover, the expression of adiponectin was reduced by eFABP4 treatment (38% decrease), with I-9 rescued the turbulence (Fig. 3h). Thus, short term/high concentration of eFABP4 injection stimulated inflammatory response in adipose tissue of C57BL/6J mice, and caused pathological transformation of adipose tissue under normal physiological state. These results suggested that eFABP4-induced adipocyte inflammation both in vitro and in vivo.

p38 MAPK is involved in eFABP4-induced lipolysis and inflammation in adipocytes

To dissect the underlying mechanisms involved in eFABP4-induced lipolysis and inflammation, we focused on p38 MAPK pathway, as it was potentially induced by eFABP4 treatment. To minimize the background activity, cells were incubated for 2 h in the presence or absence of SB203580, a well characterized inhibitor for p38 MAPK, prior to

stimulation with eFABP4. Interestingly, SB203580 did not affect basal lipolysis, but displayed a significant inhibitory effect on eFABP4-stimulated lipolysis (Fig. 4a). In accordance with the phenotype, we found that SB203580 reversed the upregulation of ser-660 p-HSL and ATGL induced by eFABP4 (Fig. 4b, c). As for inflammatory response, SB203580 also inhibited eFABP4-stimulated NF- κ B phosphorylation, as well as the expression of MCP-1, but had negligible effect on TNF- α expression (Fig. 4d, e). Taken together, lipolysis and inflammation were almost reversed by the addition of blocker specific for p38 MAPK (SB203580), indicating that p38 MAPK was involved in regulating eFABP4-mediated lipolysis and inflammation in mature adipocytes.

Discussion

With the ability of releasing various bioactive lipids and substrates as well as a variety of adipokines, adipose tissue

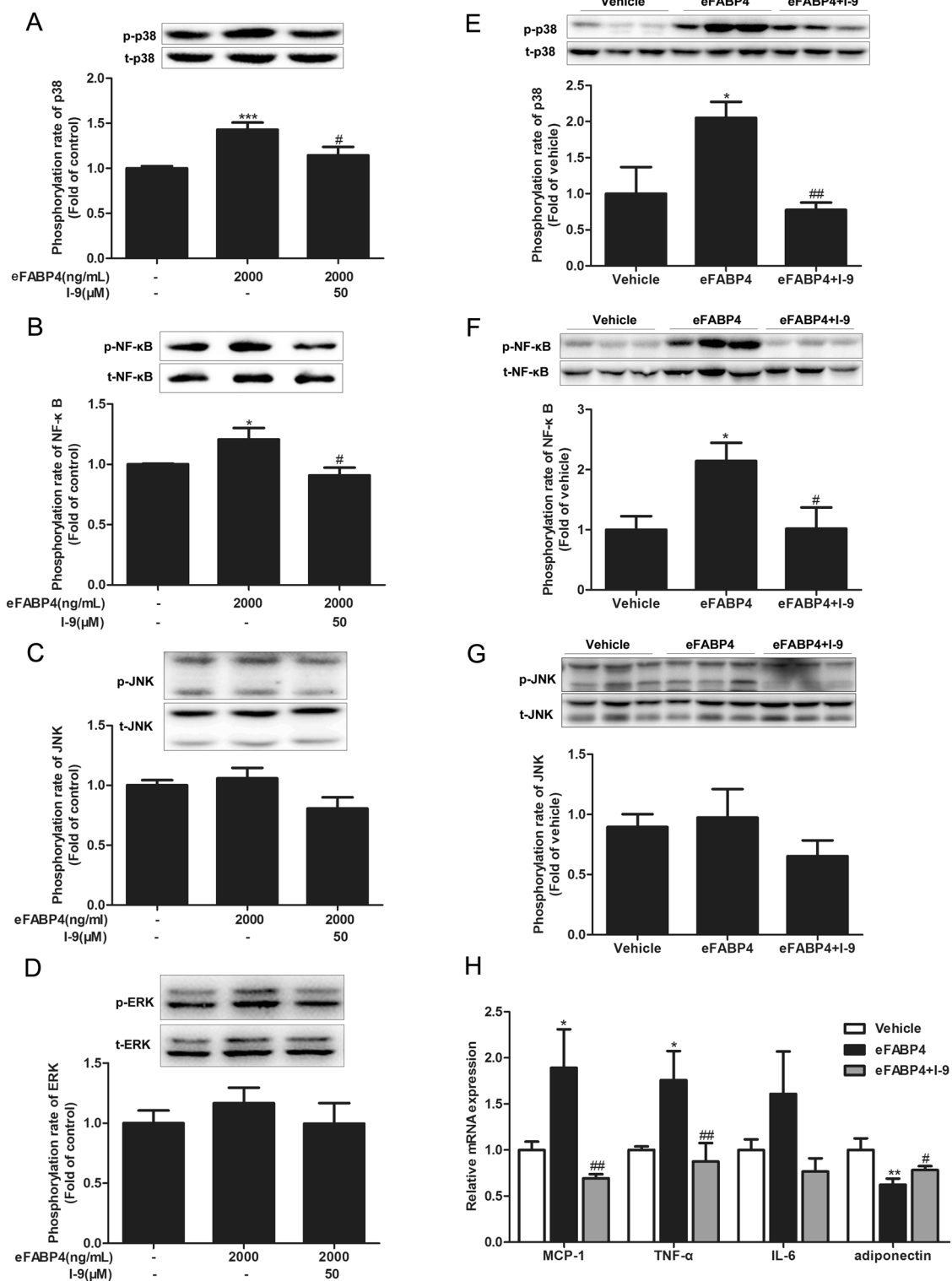


Fig. 3 eFABP4 treatment promoted inflammation in both differentiated 3T3-L1 cells and epididymal adipose tissue of C57 mice. The phosphorylation of p38, NF-κB, JNK, and ERK were detected in differentiated 3T3-L1 cells (**a–d**) and epididymal adipose tissue of C57

mice (**e–g**). **h** Gene expression of inflammatory markers and adiponectin in epididymal adipose tissue. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$ vs. the eFABP4-treated group. Values are given as mean \pm S.E.M. ($n = 3$)

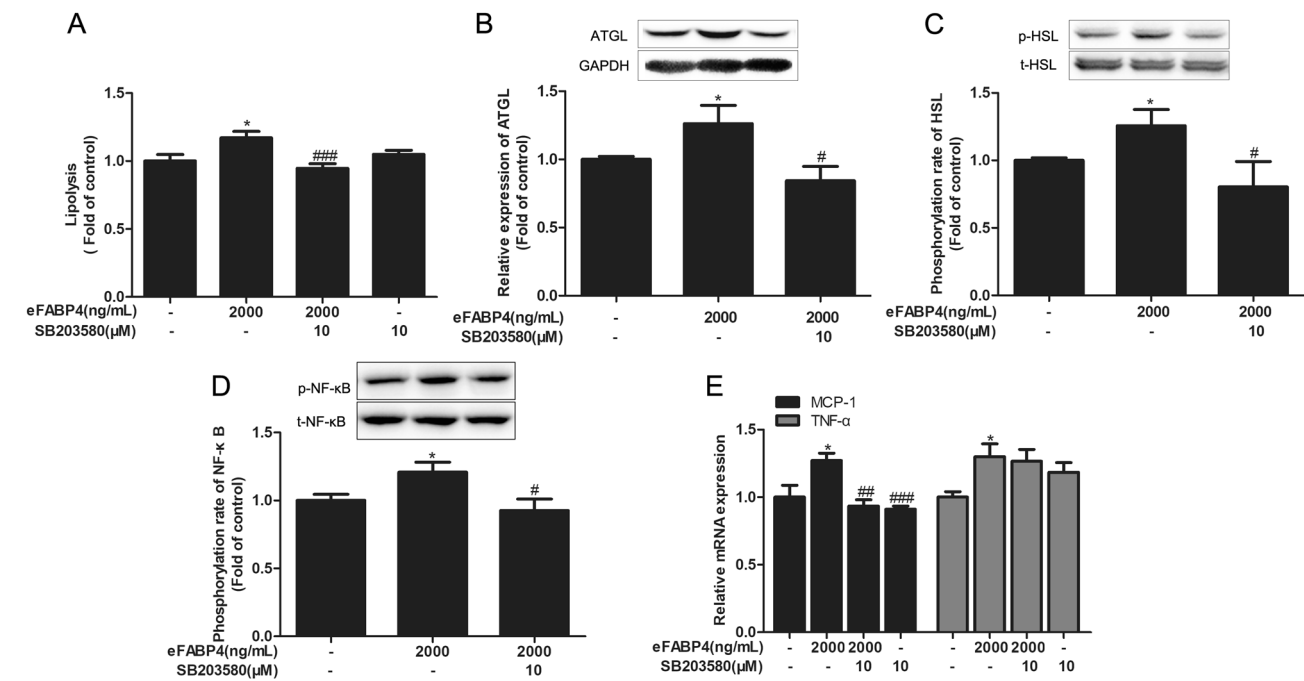


Fig. 4 p38 inhibitor SB203580 blocked eFABP4-induced lipolysis and inflammation in differentiated 3T3-L1 adipocytes. **a** Supernatant glycerol measurement. **b–d** Western blot analysis of ATGL and the phosphorylation of HSL and NF- κ B. **e** Expression of inflammatory

genes. * $p < 0.05$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the eFABP4-treated group. Values are given as mean \pm S.E.M. ($n = 3$)

has become the central focus of intracellular signaling and communication over the years [41]. A pivotal integration node among these mediators and reactions is regulated by FABP4, a lipid chaperone protein highly expressed in adipose tissue and a secreted adipokine predominantly derived from adipocytes [42]. Numerous epidemiological studies have identified circulating FABP4 as a common risk factor for metabolic disorders [24–27, 43–45], provoking questions of how circulating FABP4 levels correlate with disease, and how FABP4 interacts with target cells to mediate biological activity. Several groups have reported the function of eFABP4 on various peripheral tissues and cells, but there are at present no reports of eFABP4 on adipocytes differentiation and function. As the main source of circulating FABP4, it is significant to elucidate the influence of FABP4 on adipocytes. Hence we were committed to this research, demonstrating that eFABP4 could impair adipocyte differentiation, induce lipolysis and inflammation of mature adipocytes. More importantly, we also delineated the p38 MAPK signal involved in eFABP4-stimulated lipolysis and inflammation.

The development and maintenance of adipose tissue depend on adipocyte differentiation, which is regulated by many factors, such as glucocorticoids, cyclic AMP agonists, and insulin [46]. PPAR γ and C/EBP α , two critical transcription factors with synergetic role in supervising the entire terminal differentiation process, are involved in the differentiation of fibroblast-like pre-adipocytes into mature

adipocytes [47]. As the downstream regulatory element of PPAR γ , FABP4 has been reported to be largely induced along with adipocyte differentiation [19]. Besides, researchers have also found that FABP4 regulated adipogenesis by specifically triggering proteasomal degradation of PPAR γ and recombinant FABP4 decreased PPAR γ protein content in differentiated 3T3-L1 cells [19, 48]. Consistent with their research, we found eFABP4 negatively regulated adipogenesis during adipocyte differentiation by attenuating the expression of adipogenic transcription factors and genes, further confirming that FABP4 was not only an outcome but also a regulator of adipocyte adipogenesis. Interestingly, we found eFABP4 regulated PPAR γ not only at the posttranscriptional level, but also at the gene level, thus forming a negative feedback loop between FABP4 and PPAR γ . Adiponectin is another adipokine that is regulated by PPAR γ [49], as an insulin-sensitizing factor which can also enhance adipocyte lipid storage and maintain healthy adipose tissue expansion [50], the downregulation of adiponectin by eFABP4 was likely to disturb the physiological status of adipocytes.

FFAs released by adipose tissue during lipolysis were augmented in obesity and diabetes, and the chronic elevation of FFAs liberated by HSL could activate p38 to mediate pro-inflammatory cytokines expression in adipose tissue [51, 52]. Moreover, inflammatory cytokine such as TNF- α has been claimed to stimulate lipolysis in murine and human adipocytes [53]. We found that eFABP4

promoted adipocyte lipolysis by upregulating phosphorylation of HSL at ser-660 and ATGL level, and induced adipocyte inflammation by increasing the phosphorylation of NF- κ B and expression of MCP-1 and TNF- α . We speculated that eFABP4-induced adipocyte lipolysis and inflammation were mutually regulated to form a positive feedback loop. We also noticed the pro-inflammatory effects of eFABP4 in vivo were more obvious than that in vitro. As a more complicated circumstance, other factors may also be involved in the organism, such as the existence of adipose tissue derived macrophages (ATMs) [54]. ATMs comprise tissue resident macrophages present in adipose tissue and secrete a variety of inflammatory cytokines under certain stimulations [54]. As previously reported, eFABP4 combined with palmitic acid potentially increased the inflammatory response in macrophages [36]. The effect of eFABP4-induced inflammation in adipose tissue in vivo may at least in part attribute to its direct effect on ATMs. Moreover, considering the close association between lipolysis and FABP4 secretion, high concentration of eFABP4 treatment might simultaneously affect endogenous FABP4 secretion accompanied with lipolysis, thus disturbing the internal balance and physiological role of FABP4 in adipocytes [55].

We primarily simulated pathological states and preliminarily clarified the effects of eFABP4 and FABP4 inhibitor on normally differentiated adipocytes or adipose tissue of mice in normal physiological state, but the precise mechanisms of eFABP4/peripheral cells interaction and FABP4 inhibitor action remains unclear. eFABP4 might affect adipocytes through an autocrine/paracrine manner, and the more detailed mechanisms of eFABP4-mediated adipocyte dysfunction still needs further investigation. As previously reported, eFABP4 can be taken up into endothelial cells by interacting with plasma membrane proteins, specifically cytokeratin 1, and the pharmacological inhibition of FABP4 decreased the eFABP4-cytokeratin 1 complex formation [56, 57]. The inhibitor might interfere with the cellular uptake of eFABP4, therefore modulated its action in adipocytes. Moreover, the FABP4 inhibitor I-9 with a stronger affinity than linoleic acid might block the FABP4-fatty acid mediated disorders by competitively binding within the FABP4 fatty acid binding pocket [38].

In summary, our results demonstrate for the first time that exogenous FABP4, as a pleiotropic adipokine, interferes with adipocyte differentiation, promotes p38/HSL mediated lipolysis, and p38/NF- κ B mediated inflammation in adipocytes. Our findings give more credence to implicate FABP4 as a critical factor in coordinating cellular metabolic and inflammatory responses, and as a promising target in the pathogenesis of immunometabolic diseases, thus offering hope for the development of new therapeutic approaches for their treatments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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