Lipids (2011) 46:479–486
DOI 10.1007/s11745-011-3542-4

ORIGINAL ARTICLE

Receptor Mediated Elevation in FABP4 Levels by Advanced Glycation End Products Induces Cholesterol and Triacylglycerol Accumulation in THP-1 Macrophages

Xiao Qun Wang · Ke Yang · Yu Song He · Lin Lu · Wei Feng Shen

Received: 6 August 2010 / Accepted: 8 February 2011 / Published online: 20 February 2011

Abstract Excessive formation of advanced glycation end products (AGE) and lipid accumulation in macrophages play a pivotal role in the progression of atherosclerosis in diabetes mellitus. This study aimed to determine the molecular link between AGE-induced fatty acid binding protein 4 (FABP4) expression and macrophage lipid accumulation. AGE–BSA markedly increased macrophage FABP4 expression via engagement of RAGE, a 35-kDa transmembrane receptor that is able to bind extracellular AGE and responsible for the corresponding signal transduction, whereas knockdown of RAGE significantly reversed the FABP4 up-regulation. This effect was further paralleled with elevated intracellular total cholesterol and triacylglycerol levels. Finally, administration of FABP4 inhibitor totally abolished the increased lipid contents in response to AGE–BSA. These results indicate that FABP4 up-regulation is responsible for the enhanced macrophage lipid accumulation by AGE, which may underlie the accelerated formation of foam cells and development of atherosclerotic cardiovascular diseases in diabetic patients.

Keywords Advanced glycation end products · Receptor for advanced glycation end products · Fatty acid binding protein 4 · Lipid accumulation

X. Q. Wang · L. Lu · W. F. Shen (✉)
Department of Cardiology, Rui Jin Hospital, Jiao Tong University School of Medicine, 197 Rui Jin Road II, Shanghai 200025, People's Republic of China
e-mail: rjshenweifeng@yahoo.com.cn

X. Q. Wang · K. Yang · Y. S. He · L. Lu · W. F. Shen
Institute of Cardiovascular Diseases, Jiao Tong University School of Medicine, Shanghai 200025, People's Republic of China

Abbreviations

AGE	Advanced glycation end products
aP2	Adipocyte protein 2
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
esRAGE	Endogenous secretary RAGE
FABP4	Fatty acid binding protein 4
FABP5	Fatty acid binding protein 5
FBS	Fetal bovine serum
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
MSR	Macrophage scavenger receptor
OD	Optical density
ORO	Oil red O
oxLDL	Oxidized low-density lipoprotein
PBS	Phosphate-buffered saline
PMA	Phorbol 12-myristate 13-acetate
RAGE	Receptor for advanced glycation end products
sRAGE	Secretary RAGE
TAG	Triacylglycerol
TC	Total cholesterol

Introduction

Accelerated atherosclerosis accounts for about 80% of morbidity and mortality in patients with diabetes [1, 2]. Increased formation of advanced glycation end products (AGE) is recognized as an important player in diabetic vascular complications [3, 4]. After longstanding exposure to hyperglycemic milieu, proteins and lipids in blood and tissues undergo non-enzymatic Maillard reaction, and finally give rise to the irreversible formation of AGE [4].

AGE exert adverse effects largely through engagement of receptor for AGE (RAGE) on the cell membrane of vascular wall [5–7]. Postmortem studies have consistently shown that RAGE expression was mainly localized in macrophages within or around necrotic cores of atherosclerotic plaques, and was substantially increased in diabetic patients [8].

Macrophages actively participate in atherogenic processes, including formation of lipid-laden foam cells and atheromatous lipid cores [9]. FABP4, also known as adipocyte protein 2 (aP2), is highly expressed in macrophages [10]. It functions in cellular lipid metabolism, cholesterol trafficking and other biologic responses [11, 12]. Expression of FABP4 in THP-1 monocytes/macrophages was greatly increased upon treatment with phorbol 12-myristate 13-acetate (PMA) and oxidized low-density lipoprotein (oxLDL), while transformation of THP-1 monocytes into foam cells was significantly suppressed by FABP4 inhibitor, a small synthetic molecule designed to competitively prevent FABP4 from binding fatty acids [13, 14]. Likewise, inhibition of FABP4 expression either by macrophage-specific gene deficiency or administration of FABP4 inhibitor in vivo led to a protection from the development of atherosclerosis [15]. Iwashima et al. [6] observed that macrophage lipoprotein receptors, including CD36, macrophage scavenger receptor (MSR) class A and lectin-like oxLDL receptor 1 (LOX-1) were up-regulated in response to AGE stimulation, whereas key proteins localized on the cell membrane that mediate macrophage cholesterol efflux, including ABCA1 and ABCG1, were down-regulated or destabilized [16, 17].

It is still unclear, however, whether macrophage FABP4 remains, equally or to a greater extent, an active pro-atherogenic participator in the context of diabetes where AGE are greatly enriched. In this study, we tested the hypothesis that AGE-induced FABP4 expression in THP-1 macrophages was potentially associated with an increase in intracellular lipid levels. The assessment of lipid contents after FABP4 inhibitor administration was also performed.

Materials and Methods

Cell Culture

Human monocytic leukemia THP-1 cells were obtained from ATCC and cultured in Gibco RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 50U/ml streptomycin (Gibco, Auckland, New Zealand) at 37 °C in 5% CO_2. THP-1 monocytes were differentiated into macrophages by induction of 100nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, MO) for 48 h. Cells were then incubated in the presence or absence of bovine serum albumin (BSA; fatty acid free, catalog no. 126575; Calbiochem, CA) or AGE–BSA (catalog no. 121800; Calbiochem, CA) for the indicated time intervals. FABP4 inhibitor (Calbiochem, CA) dissolved in dimethyl sulfoxide (DMSO) at the indicated concentrations was administrated for study purpose.

Transfection of RAGE-Specific siRNA

Transient transfection of THP-1 macrophages with RAGE-specific and negative control siRNA (Ambion CA) was scheduled as follows: Silencing RAGE expression was performed using human RAGE-specific siRNA and siRNA transfection reagent (Lipofectamine 2000, Invitrogen, CA) to macrophages. Macrophages grown in 6-well plates were transfected with various concentrations of siRNA according to the manufacturer's protocol. The RAGE-specific siRNA (100 pM) for reducing RAGE expression was added 48 h before AGE stimulation; macrophages were then exposed to BSA or AGE–BSA (Calbiochem CA) for 24 or 48 h for RNA or protein analysis and ORO-staining, respectively.

Assessment of Intracellular Lipid Contents

Cultured and transfected macrophages were washed twice in phosphate-buffered saline (PBS, Gibco, Auckland, New Zealand) and then fixed in 4% paraformaldehyde for 30 min. After rinsing with ddH2O, macrophages were incubated with filtered Oil Red O solution (0.6 g/L ORO in 60% isopropanol) (ORO, Sigma-Aldrich, MO) for 10 min and with 1 mL of Mayer's hemalum (Merck, Darmstadt) for 1 min. After rinsing with PBS four times, intracellular lipid droplets were evaluated under Olympus DP-71 microscope. Spectrophotometric quantification of the staining was performed as described previously [18]. Briefly, stained oil droplets were dissolved in 100% isopropanol for 10 min. Then optical density was measured at 500 nm and equalized with the cell numbers estimated by microscope. Quantifications of intracellular total cholesterol and triacylglycerol levels were performed following the protocols from the manufacture (Biovision, CA) and analyzed with a microtiter plate reader (Ex/Em = 538/587 nm). Total cholesterol and triacylglycerol concentrations were expressed as micrograms per milligram protein and nanomoles per milligram protein sample, respectively.

Quantitative Real-Time PCR Analysis

Total RNA was isolated using Trizol reagent (Invitrogen, CA). For reverse transcription, 1 μg of the total RNA was converted to first strand complementary DNA in 20-μl reactions using a reverse transcription kit (Promega, WI). Quantitative real-time PCR analysis was performed

(StepOne, Applied Biosystems) using SYBR Green (Takara, Dalian, China). The thermal cycling program was 10 s at 95 °C for enzyme activation and 40 cycles of denaturation for 5 s at 95 °C, 31 s 60 °C for annealing and extension. The comparative cycle threshold (CT) method was used to determine relative mRNA expression of genes as normalized by β-actin housekeeping gene. Primers used are as follows: 5′-TGGGATGGAAAATCAACC-3′ for FABP4-F, 5′-TCTCTCATAAACTCTCGTGG-3′ for FABP4-R, 5′-GGCAGAAAAACTCAGAC-3′ for FABP5-F, 5′-GACACACTCCACCACTAA-3′ for FABP5-R, 5′-GCTGGTGTTCCCAATAA-3′ for AGER-F, 5′-AGTGTGAAGAGCCCTGT-3′ for AGER-R, 5′-CGTGGACATCCGCAAAG-3′ for β-actin-F, and 5′-TGGAAGGTGGACAGCGA-3′ for β-actin-R.

Western Blot

Total proteins were prepared by standard procedures and assessed by microplate protein assay (BSA; Pierce, IL). Thirty micrograms of protein per sample and known molecular weight markers were loaded onto a 12% SDS-polyacrylamide gel. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to PVDF membranes (Millipore, MA). The blocked membranes (5% nonfat milk in TBS buffer containing 0.1% Tween 20) were incubated with anti-FABP4 (1:1000; Santa Cruz Biotechnology, CA), anti-RAGE (1:1000; Abcam, MA) and anti-β-actin antibody (1:1000; Cell Signaling Technology, MA) for overnight at 4 °C. The membranes were then treated with horseradish peroxidase-conjugated rabbit anti-goat, goat anti-rabbit or goat anti-mouse antibodies (1:5000; Bio-Rad, Hercules, CA). After washing, immunodetection analysis was accomplished using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA).

Statistical Analysis

Data are expressed as means ± SEM. The mean values for biochemical measurements from each group were compared using Student's t test. A $P < 0.05$ was considered statistically significant.

Results

AGE–BSA-Induced Up-Regulation of FABP4 Expression

After incubation with AGE–BSA for 24 h at various concentrations (from 25 to 200 μg/ml), FABP4 expression was substantially up-regulated, and reached the maximum level

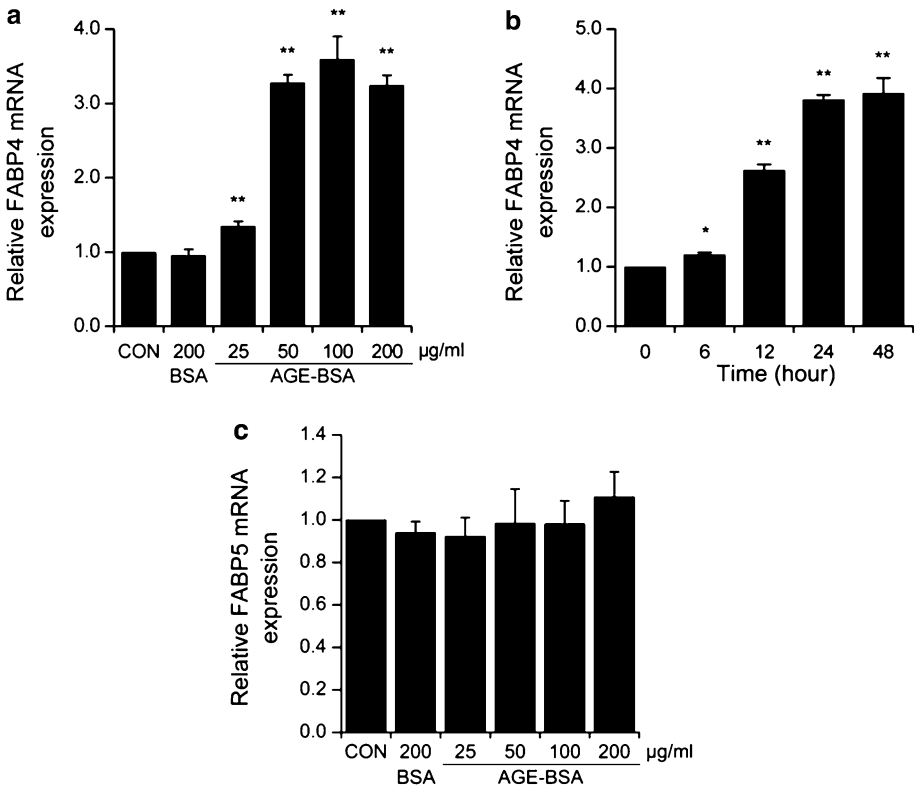

Fig. 1 Up-regulation of FABP4 mRNA expression by AGE–BSA. **a**, **c** THP-1 macrophages were treated with indicated doses of BSA or AGE–BSA for 24 h. **b** Macrophages were either untreated or treated with 100 μg/ml AGE–BSA for various time intervals. FABP4 and FABP5 mRNA levels were detected by quantitative real-time PCR; data are expressed relatively to untreated cells, arbitrarily set at the level of 1, and are the means ± SEM of at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ when compared with untreated cells

at 100 μg/ml (3.59 ± 0.53-fold vs. con; $P = 0.001$), whereas BSA did not affect FABP4 expression significantly (0.95 ± 0.14-fold vs. con, $P = 0.577$; Fig. 1a). A 3.9-fold increase in FABP4 expression was observed by 48 h of incubation ($P = 0.0003$; Fig. 1b). FABP5, another fatty acid binding protein expressed in macrophages, was not affected by AGE (Fig. 1c).

RAGE Mediates AGE–BSA-Induced FABP4 Expression

AGE–BSA moderately increased RAGE expression by 1.2-fold in cells transfected with non-specific negative control siRNA ($P = 0.010$, Fig. 2a). After gene silencing of RAGE, AGE exposure no longer affected FAPB4 expression both at mRNA (1.38 ± 0.24-fold vs. BSA, $P = 0.069$; Fig. 2b) and protein (Fig. 2c) levels when compared with negative control-treated groups (2.78 ± 0.36-fold vs. BSA, $P = 0.001$).

AGE–BSA-Induced Macrophage Lipid Accumulation

Oil red O (ORO) staining of lipid droplets was most evident at 100 and 200 μg/ml of AGE–BSA (Fig. 3a, b). A similar trend was also obtained for intracellular TC (100 μg/ml AGE–BSA: 37.20 ± 2.52 μg/mg, $P = 0.0002$; 200 μg/ml AGE–BSA: 29.89 ± 4.67 μg/mg, $P = 0.0066$ vs. con: 15.10 ± 1.61 μg/mg; Fig. 3c) and TAG levels (100 μg/ml AGE–BSA: 4.35 ± 1.35 nmol/mg, $P = 0.0252$; 200 μg/ml AGE–BSA: 2.89 ± 0.75 nmol/mg, $P = 0.0463$ vs. con: 1.64 ± 0.08 nmol/mg; Fig. 3d). RAGE knockdown markedly reduced lipid contents in response to AGE exposure (TC, 21.92 ± 3.09 μg/mg vs. BSA: 17.23 ± 2.20 μg/mg, $P = 0.0980$; TAG, 5.64 ± 1.43 nmol/mg vs. BSA: 1.85 ± 1.10 nmol/mg, $P = 0.0220$) when compared with negative control-treated cells, as demonstrated by ORO staining (Fig. 3e, f), TC (Fig. 3g) and TAG (Fig. 3h) quantification.

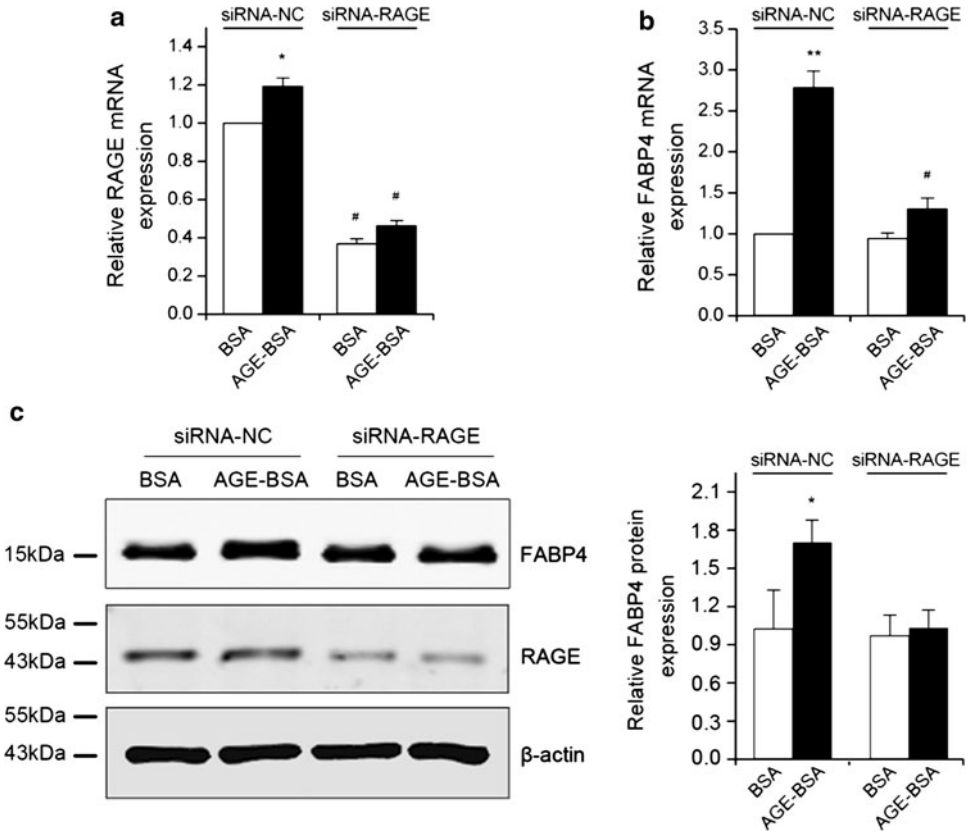

Fig. 2 Effect of RAGE knockdown by siRNA transfection on FABP4 expression. Macrophages were transfected with negative control or RAGE specific siRNA for 48 h and exposed to BSA or AGE–BSA, respectively, at the concentration of 100 μg/ml. Then mRNA expression levels of RAGE (**a**) and FABP4 (**b**) were determined using quantitative real-time PCR; data are expressed relatively to untreated cells, arbitrarily set at the level of 1, and are the means ± SEM of at least three independent experiments. **c** Protein expression levels of FABP4 and RAGE in each group were analyzed by Western blot. β-actin demonstrated equal loading. The right panel shows the average densitometric analysis of three independent experiments. Data are expressed in arbitrary units. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with BSA-treated cells in the same group. $^{\#}P < 0.01$ versus cells with the same BSA or AGE–BSA treatment in negative control group

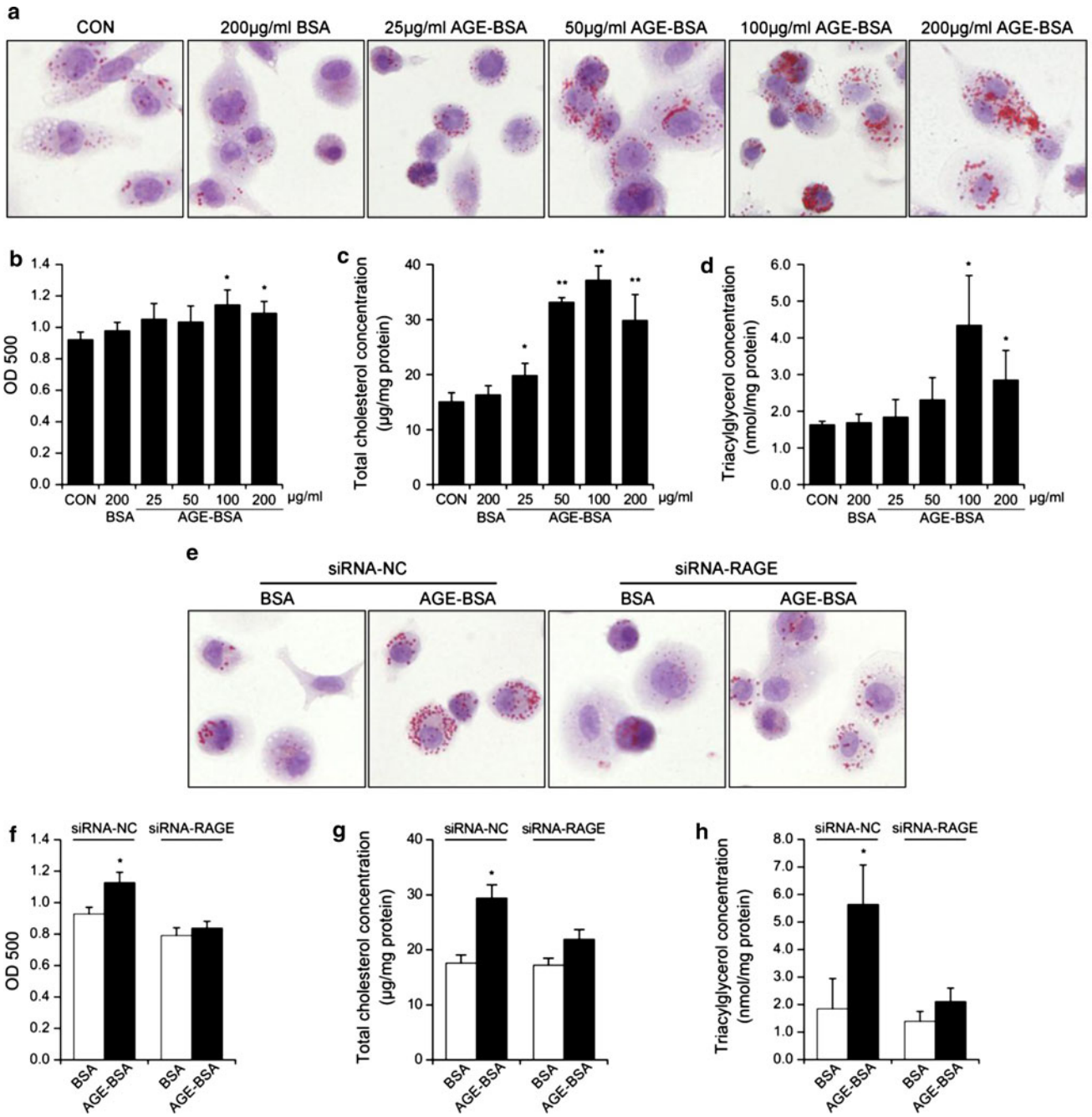

Fig. 3 Up-regulation of lipid contents by AGE–BSA via RAGE. **a**–**d** Macrophages were either untreated or treated with indicated doses of BSA and AGE–BSA for 48 h. **e**–**h** Macrophages were transfected with negative control or RAGE specific siRNA for 48 h and then exposed to BSA or AGE–BSA, respectively, at the concentration of 100 μg/ml. **a**, **e** Intracellular lipid droplets were observed through light microscope after ORO staining; pictures acquired at the magnification of 400× and processed using identical conditions are representative of three independent experiments. **b**, **f** Spectrophotometric quantifications of ORO staining by optical density at 500 nm were shown. Intracellular total cholesterol (**c**, **g**) and triacylglycerol (**d**, **h**) concentrations were determined using a fluorometric method; data are presented as micrograms of total cholesterol per milligrams of protein or nanomoles of triacylglycerol per milligrams of protein ± SEM of three independent experiments. $^{*}P < 0.05$ versus untreated cells (**b**, **c**, **d**) or BSA-treated cells in the same group (**f**, **g**, **h**). $^{**}P < 0.01$ versus untreated cells

Changes After Incubation with FABP4 Inhibitor

After incubation with FABP4 inhibitor for 48 h, AGE-induced lipid accumulation in macrophages was substantially suppressed and reached the maximum effect at 40 μM as demonstrated by ORO staining (Fig. 4a, b), TC (14.92 ± 1.54 μg/mg vs. BSA: 14.56 ± 0.81ug/mg, $P = 0.7356$; Fig. 4c) and TAG (0.79 ± 0.35 nmol/mg vs. BSA:

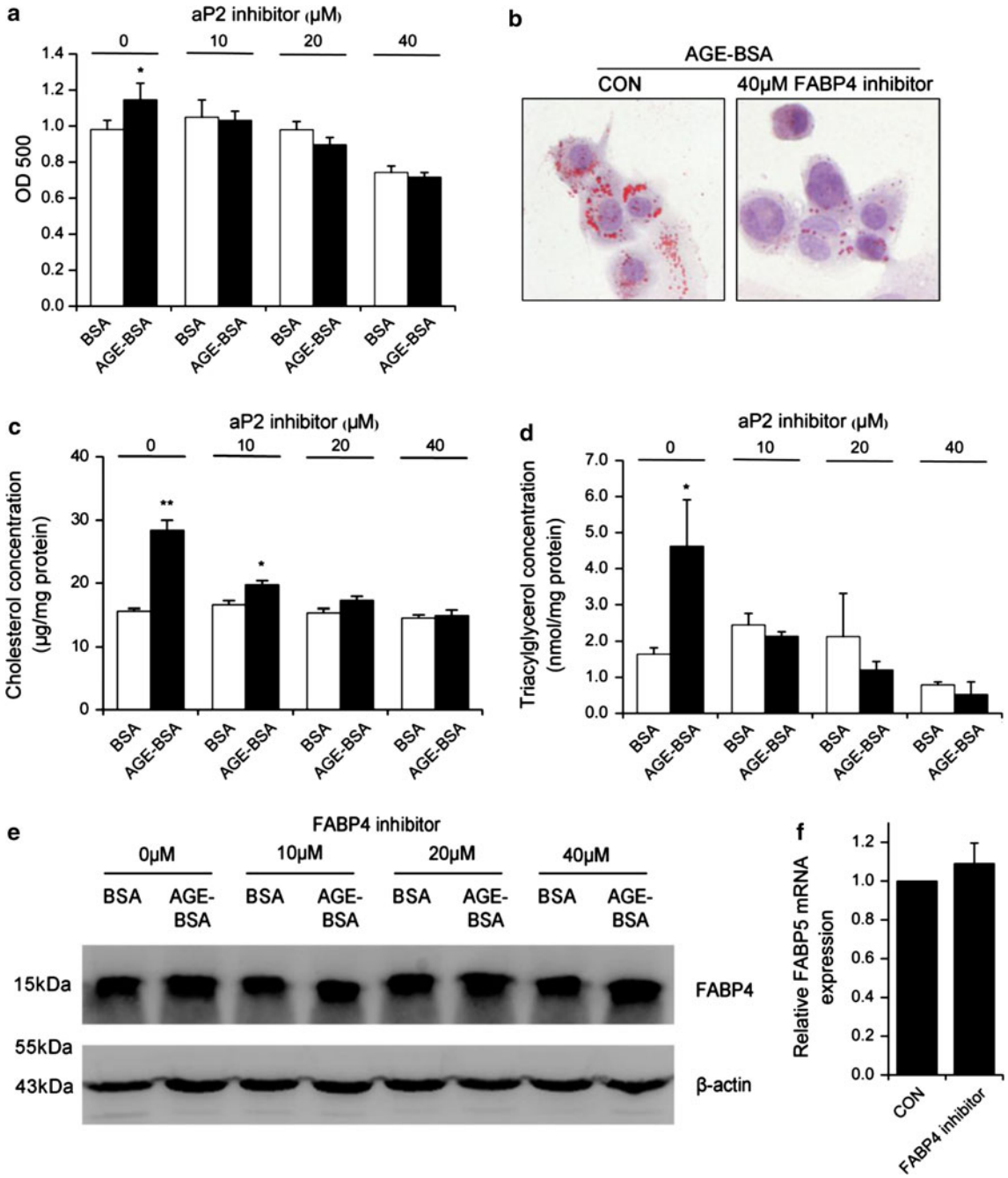

Fig. 4 Effect of FABP4 inhibitor on AGE–BSA-induced lipid accumulation. Macrophages were treated with 100 μg/ml BSA or AGE–BSA in the absence or presence of indicated doses of FABP4 inhibitor for 48 h. **a** Intracellular cellular lipid droplets were stained with ORO and quantified by optical density at 500 nm or **b** observed through light microscope; pictures acquired at the magnification of 400× and processed using identical conditions are representative of three independent experiments. Intracellular total cholesterol (**c**) and triacylglycerol (**d**) concentrations were determined using a fluorometric method; data are presented as micrograms of total cholesterol per milligrams of protein or nanomoles of triacylglycerol per milligrams of protein ± SEM of three independent experiments. **e** FABP4 protein expression levels in each group were analyzed by Western blot. *β*-actin demonstrated equal loading. **f** THP-1 macrophages were treated with or without 40 μM FABP4 inhibitor for 48 h. FABP5 mRNA levels were detected by quantitative real-time PCR; data are expressed relatively to untreated cells, arbitrarily set at the level of 1, and are the means ± SEM of at least three independent experiments. $*P < 0.05$; $**P < 0.01$ versus BSA-treated cells in the same group

0.52 ± 0.24 nmol/mg, $P = 0.2656$; Fig. 4d) quantification when compared with macrophages without FABP4 treatment. AGE–BSA up-regulated FABP4 protein levels irrespective of FABP4 inhibitor at each concentration (Fig. 4e). FABP5 expression was not affected by FABP4 inhibitor (Fig. 4f).

Discussion

In THP-1 macrophages, AGE-induced FABP4 expression via a RAGE-dependent signaling pathway resulted in increased intracellular lipid levels, which could be attenuated by FABP4 inhibitor.

AGE are increasingly regarded as an indispensable factor for severity and progression of atherosclerosis in patients with diabetes [8]. AGE-albumin, a further glycation modified protein derived from Amadori adduct exerts more severe pathogenic influence. Elevated serum AGE-albumin and decreased serum endogenous secretary RAGE (esRAGE) levels highly correlated with the extent of angiographic severity in patients with type 2 diabetes [19, 20]. In contrast, in vivo studies showed that administration of soluble RAGE (sRAGE), another truncated form of RAGE acting as a decoy for AGE, completely suppressed diabetic atherosclerosis in glycemia- and lipid-independent manners [21]. Other approaches utilizing inhibitors for AGE formation, including aminoguanidine and an AGE cross-link breaker ALT-711, also led to a striking reduction in atherosclerotic lesions in diabetic apoE-deficient mice [22].

Despite the important contribution of AGE to the accelerated atherosclerosis in diabetes, the specific molecular mechanisms in response to AGE within a macrophage (a central player in atherogenesis) remain unclear. In this study, we discovered that exposure of THP-1 macrophages to AGE was time-dependently associated with a significant elevation of FABP4 expression, reaching its maximum level when 100 μg/ml AGE–BSA was used. At the same time, the enhanced expression of FABP4 was paralleled with increases in intracellular TC and TAG levels. RAGE-specific gene silencing led to reversions both in FABP4 expression and lipid contents, suggesting that AGE-elicited atherogenic effects in macrophages were, at least partly, RAGE-dependent. Finally, this increase in lipid levels was totally abolished by simultaneous administration of FABP4 inhibitor.

Our study is the first to show that macrophage FABP4 expression, a critical participant in atherogenesis, is further enhanced in the context of diabetes. In an animal experiment, Gerrity et al. [2] observed that FABP4 deficiency protected against the development of insulin resistance, diabetes, and atherosclerotic cardiovascular disease. Recent population studies revealed that genetic variations at the FABP4 locus in humans led to lowered serum triglyceride levels, and a markedly reduced risk of coronary heart disease in type 2 diabetes [23]. These anti-atherosclerotic effects were mainly attributed to the improvement of glucose control, insulin resistance and dyslipidemia [24, 25]. In this study, we demonstrated that exposure of macrophages to AGE was capable of increasing intracellular FAPB4 levels and lipid contents, similar to that when transforming macrophages to foam cells in the presence of oxLDL stimulation [14]. These observations suggest that excessive formation of AGE could render diabetic patients under high risk of developing atherosclerosis like those with dyslipidemia. [26–28].

Furthermore, our results imply that FABP4 might be a potential therapeutic target for the treatment of diabetic patients with atherosclerosis. In this study, mild doses of FABP4 inhibitor (10–20 μM) greatly attenuated AGE–BSA elicited macrophage lipid accumulation, whereas lipid levels in the absence of AGE were not affected. These findings indicate that FABP4 inhibitor may not disturb physiological lipid metabolism in macrophages, but, on the contrary, suppresses exclusively pathological excessive lipid accumulation.

This study does not specify the mechanisms by which lipid contents and trafficking are affected by AGE besides the elevated FAPB4 level. Deficiency of FABP4 enhanced CD36-mediated lipoprotein entry and, at the same time, activated ABCA1-dependent lipid efflux to a greater extent, thereby lowering intracellular lipid contents [29]. Likewise, AGE stimulation on macrophages could increase SRA-1 and CD36 and decrease ABCG1 protein levels [6, 16, 17]. Thus, enhanced expression of FABP4 by AGE might extensively affect lipid influx and efflux, directly or indirectly, at the same time to increase lipid accumulation. Further studies including analysis of lipid trafficking in macrophages from FABP4-deficient mice and blocking lipoprotein transporters by their specific siRNA or antibodies, are needed to elucidate the mechanisms.

In conclusion, this study demonstrates a causal molecular link between AGE and FABP4 in macrophage lipid accumulation. FABP4 inhibitor may be useful for suppressing the atherosclerotic process in patients with diabetes.

Acknowledgments This work was supported by grant from Chinese National Nature Science Foundation (No. 30871084).

Conflict of interest All authors have no conflict of interest.

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