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New insights into circulating FABP4: Interaction with cytokeratin 1 on endothelial cell membranes

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ABSTRACT

Fatty acid-binding protein 4 (FABP4) is an adipose tissue-secreted adipokine that is involved in the regulation of energetic metabolism and inflammation. Increased levels of circulating FABP4 have been detected in individuals with cardiovascular risk factors. Recent studies have demonstrated that FABP4 has a direct effect on peripheral tissues, specifically promoting vascular dysfunction; however, its mechanism of action is unknown. The objective of this work was to assess the specific interactions between exogenous FABP4 and the plasma membranes of endothelial cells. Immunofluorescence assays showed that exogenous FABP4 localized along the plasma membranes of human umbilical vein endothelial cells (HUVECs), interacting specifically with plasma membrane proteins. Anti-FABP4 immunoblotting revealed two covalent protein complexes containing FABP4 and its putative receptor; these complexes were approximately 108 kDa and 77 kDa in size. Proteomics and mass spectrometry experiments revealed that cytokeratin 1 (CK1) was the FABP4-binding protein. An anti-CK1 immunoblot confirmed the presence of CK1. FABP4-CK1 complexes were also detected in HAECs, HCASMCs, HepG2 cells and THP-1 cells. Pharmacological FABP4 inhibition by BMS309403 results in a slight decrease in the formation of these complexes, indicating that fatty acids may play a role in FABP4 functionality. In addition, we demonstrated that exogenous FABP4 crosses the plasma membrane to enter the cytoplasm and nucleus in HUVECs. These findings indicate that exogenous FABP4 interacts with plasma membrane proteins, specifically CK1. These data contribute to our current knowledge regarding the mechanism of action of circulating FABP4.

1. Introduction

Fatty acid-binding protein 4 (FABP4; adipocyte-FABP; aP2) is a member of the family of intracellular fatty acid-binding proteins (FABPs), which are ~15 kDa in weight and 126–134 amino acids in length [1,2]. FABPs are expressed abundantly (1–5% of cytosolic proteins) in cells involved in active lipid metabolism. Members of the FABP family exhibit unique tissue-specific expression patterns and are named according to the tissues in which they were first identified [3]. FABPs are capable of binding a variety of hydrophobic ligands, such as long-chain fatty acids, eicosanoids, leukotrienes and prostaglandins [4–6]. However, the divergent sequences of the members confer subtle differences in their ligand-binding properties and may also indicate different protein–protein interaction partners depending on the cellular context. Indeed, it has been shown that intracellular adipocyte-, epithelial-, and heart-type FABPs interact with hormone-sensitive lipase, whereas the intestinal and liver isoforms do not [3–7]. It is also known that FABP4 interacts with Janus Kinase 2 in a fatty acid-dependent manner, establishing a new role for FABP4 as a fatty acid sensor that affects cellular metabolism via protein–protein interactions [8]. FABP4 is highly expressed in adipocytes and macrophages and has a role in lipid metabolism in both cell types [8]. FABP4 expression has also been reported in endothelial cells [9]. The biological relevance of FABP4 is underscored by the findings that FABP4 knockout (FABP4 $^{-}/^{-}$) mice exhibit marked protection against insulin resistance, atherosclerosis, fatty liver disease, and asthma [5,10–14]. Consistent with these studies, a small-molecule inhibitor of FABP4 (BMS309403) has been found to be an effective therapeutic agent for the treatment of atherosclerosis and type 2 diabetes mellitus (T2DM) in mouse models [15]. FABP4 is secreted from adipocytes via a non-classical, calcium-dependent mechanism [16] that is regulated by lipolytic pathways [17]. It has been demonstrated that adipocyte lipases, specifically adipose triglyceride lipase (and, to

Abbreviations: CK1, cytokeratin 1; FABP4, fatty acid-binding protein 4; FABP4-His, polyhistidine-tagged fatty acid-binding protein 4; FABP, fatty acid-binding proteins; T2DM, type 2 diabetes mellitus; NO, nitric oxide

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a lesser extent, hormone-sensitive lipase), are involved in the regulated secretion of FABP4 [18]. In the last several years, much effort has been focused on determining the role of circulating FABP4. We and other authors have shown that FABP4 levels are increased in obesity, metabolic syndrome, T2DM, and familial combined hyperlipidemia and lipodystrophy syndromes. In addition, FABP4 levels are closely correlated with adverse lipid profiles and insulin resistance [19–25]. FABP4 serum levels independently predict the risks of developing metabolic syndrome, T2DM and atherosclerotic disease [24,26–28]. A recent study has found that circulating FABP4 levels are inversely associated with peripheral reactive hyperemia, a surrogate marker of endothelial dysfunction [29]. In addition, it has been demonstrated that increased levels of FABP4 in atherosclerotic lesions are associated with unstable plaque phenotypes [30,31]. Extracellular FABP4 also directly decreases the contractility of myocardial muscle cells, suggesting that its release into the bloodstream could directly affect certain peripheral cells and tissues [32]. Recent results from our group have shown that along with a decrease in eNOS expression, extracellular FABP4 increases the expression of vascular cell adhesion protein 1 (VCAM1), E-selectin and leukocyte adhesion to endothelial cells; these results suggest that FABP4 has a global effect on endothelial function [33]. Furthermore, FABP4 directly affects the migration and proliferation of human artery coronary smooth muscle cells (HCASMCs). This result suggests a role for FABP4 in vascular remodeling that is mediated primarily through a MAPK-dependent pathway, which activates the transcription factors c-jun and c-myc in HCASMCs [34].

These data prompted us to investigate the interactions of exogenous FABP4 with plasma membrane proteins in endothelial cells. Here, based on the results of immunofluorescence and cross-linking immunoblot experiments, we report the cell-surface localization of exogenous FABP4 protein complexes. Proteomics analysis subsequently revealed that FABP4 binds specifically with cytokeratin 1 (CK1).

2. Materials and methods

2.1. Cell culture, treatment and protein extracts

Human umbilical vein endothelial cells (HUVECs) (passage 3) and human aortic endothelial cells (HAECs) were cultured in Medium 200 supplemented with 2% low serum growth supplement (LSGS) and 1% gentamicin/amphotericin (GIBCO®, Oregon, OR, USA). Human coronary aortic smooth muscle cells (HCASMCs) were cultured in Medium 231 supplemented with 2% smooth muscle growth supplement (SMGS) and 1% gentamicin/amphotericin (GIBCO®). Monocyte-derived THP-1 macrophages (DSMZ, Braunschweig, Germany) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (BioWest, Kansas City, MO, USA). HepG2 cells (ATTC, Manassas, VA, USA) were cultured in MEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% non-essential amino acids (NEAA) (BioWest).

The cells were incubated with or without human recombinant FABP4 (100 ng/mL) (BioVendor, Heidelberg, Germany) or human recombinant polyhistidine-tag FABP4 (FABP4-His) (Enzo Life Sciences, San Diego, CA, USA) for the indicated times. In some experiments, HUVECs were incubated with the FABP4 inhibitor BMS309403 {2-[2′-(5-ethyl-3,4-diphenyl-1H-pyrazol-1-yl)biphenyl-3-yloxy] acetic acid} at 10 μM (Calbiochem, San Diego, CA, USA). Dimethyl sulfoxide (DMSO) was used as a solvent control (Sigma-Aldrich).

Cell plasma membrane lysates were obtained as previously reported [35]. Briefly, the cells were incubated with cold buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM Ca^{2+}, 1% Triton X-100, 1% NP40, and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and kept on ice for 15 min. After incubation, the lysates were centrifuged at 16,000 × g for 15 min, and the supernatant was collected. Nuclear and cytoplasmic protein extracts were obtained as previously described [34].

2.2. Immunoblotting

Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Electrophoresis and immunoblot analyses were performed using the NuPAGE protein analysis system (Invitrogen Life Technologies, Carlsbad, CA, USA). The membranes were incubated with anti-FABP4 (R&D Systems, Minneapolis, MN, USA), anti-His-tag (Abcam, Cambridge, MA, USA), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD31 (DAKO, Glostrup, Denmark) and anti-cytokeratin 1 (CBL266) (Merck Millipore, Billerica, MA, USA) antibodies. The antigen-antibody complexes were detected by incubating the membrane with HRP-conjugated anti-IgG antibodies (DAKO). The bands were visualized using a ChemiDoc Image System and quantified with Image Lab analysis software (Bio-Rad). The relative levels of FABP4 were quantified after normalization with CD31 or actin levels. All of the values were expressed in arbitrary units (AU). The molecular weights of the bands were assigned by comparing the band weights with BenchMark™ Protein Ladder (Invitrogen Life Technologies) using ImageQuant TL v7.0 v (GE Healthcare, Wauwatosa, WI, USA).

2.3. Immunofluorescence microscopy

For the colocalization experiments, HUVECs were incubated in chambered slides (Nunc, Roskilde, Denmark) with or without FABP4, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at 4 °C and washed at each step three times with DPBS (GIBCO®) for 5 min. The cells were then incubated overnight at 4 °C with anti-FABP4 and anti-CD31 antibodies and further incubated with Alexa Fluor® 488 anti-goat and Alexa Fluor® 532 anti-mouse antibodies (Invitrogen Life Technologies) for 3 h at RT. The antibodies were diluted in the blocking solution (DPBS, 2% FBS, and 0.1% BSA). Confocal immunofluorescence images were captured with a Nikon Eclipse TE2000-E microscope and processed with EZ-C1 3.40 software (Nikon, Chiyoda, Tokyo, Japan).

For the internalization experiments, the cells were permeabilized after fixation with a solution containing DPBS, 2% FBS, 0.1% BSA, and 0.1% Triton X-100 for 5 min at RT. The cells were then incubated with blocking solution for 20 min at RT. The cells were incubated overnight at 4 °C with an anti-FABP4 antibody and further incubated with an Alexa Fluor® 488 anti-goat antibody for 3 h at RT. The cells were then incubated with the nuclear stain DAPI (4′,6-diamidino-2-phenylindole, dilactate) and CellMask™ Deep Red Plasma Membrane Stain (Invitrogen Life Technologies) for 5 min. Immunofluorescence images were captured with an Olympus IX71 inverted microscope, processed with Cell^F Software (Olympus, Shinjuku-ku, Tokyo, Japan) and quantified with ImageJ (Fiji, Madison, WI, USA).

2.4. HUVEC crosslinking

After incubation with or without FABP4-His, the HUVECs were cross-linked with 2% formaldehyde in DPBS at RT for 30 min, as previously reported [36,37]. The cells were washed once in DPBS, and HUVEC plasma membrane protein extraction was performed as mentioned above.

2.5. Polyhistidine-tagged protein purification

Plasma membrane proteins from HUVECs that were incubated with or without FABP4-His were purified according to the instructions provided with the HisPur™ Cobalt Purification Kit, which was purchased from Pierce (Rockford, IL, USA).

2.6. Ligand screening assay

Plasma membrane proteins from HUVECs incubated with or without FABP4-His were analyzed with the FABP4 Inhibitor/Ligand Screening Assay Kit (Cayman Chemical Company, Ann Harbor, MI, USA) to detect

FABP4 ligands. Briefly, the protein lysates were incubated with a fluorescent probe that fluoresces when bound to FABP4 and recombinant FABP4 (10 mM). Any FABP4 ligand can displace the fluorescent probe, thereby reducing fluorescence. We used arachidonic acid (1.28 mM), a known ligand of FABP4, as a positive control. The binding of the fluorescent probe to FABP4 was monitored by measuring excitation at 370 mm and emission at 475 mm using a Synergy HT fluorometer (BioTek, Winooski, VT, USA). The obtained results were presented as the percent fluorescence of each sample (plasma membrane proteins) relative to the fluorescence maximum (FABP4 + fluorescence probe).

2.7. Small interfering RNA (siRNA) transfection

To knock down FABP4 expression, ON-TARGETplus siRNAs targeting human FABP4 (J-008,853–08–0005) and GAPDH (D-001,830–01–05) as a control were purchased from Dharmacon (Lafayette, CO, USA). The cells were transiently transfected using Lipofectamine® RNAiMax (Invitrogen Life Technologies) and analyzed after 72 h.

2.8. Quantitative real-time PCR

Total RNA was isolated from the cells using the ABI PRISM 6100 Nucleic Acid PrepStation kit (Applied Biosystems, Carlsbad, CA, USA). The absorbance at 260 nm was used to determine the RNA concentration, and the 260/280 ratio was used to determine the RNA quality. Total RNA (0.5 μg) was reverse transcribed to cDNA using random hexamers and SuperScript II (Invitrogen Life Technologies), following the manufacturer's protocol. Pre-designed and validated TaqMan Gene Expression Assay (Life Technologies) primers and probes were obtained for FABP4 (Hs00609791_m1) and 18S (Hs99999901_s1) and used for real-time PCR amplification. The mRNA expression of each gene and sample was calculated using the recommended 2-ΔΔCt method. Untreated cells were considered as controls in this experiment. 18S was used as a housekeeping gene to normalize the results for the gene of interest.

2.9. Proteomics sample preparation and MS analysis

Concentrated HUVEC plasma membrane proteins were incubated with or without FABP4-His, and impurities were removed using a 2D clean-up kit (GE Healthcare) according to the manufacturer's instructions. After quantification, the samples were separately labeled with either Cy3 or Cy5 according to the manufacturer's protocol. The labeled samples were mixed and combined for MS analysis.

First-dimension separation was performed with an IPGphor isoelectric focusing (IEF) unit (GE Healthcare), in which the samples were loaded onto 7-cm, pH 3–10 immobilized pH gradient (IPG) strips with passive rehydration for 16 h. This step was followed by isoelectric focusing for a total of 7000 V/h. The strips were immediately equilibrated in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 75 mM Tris Cl, pH 8.8, and 0.005% bromophenol blue) for 15 min with 1% (*w*/*v*) DTT. The strips were then equilibrated for 15 min with SDS buffer with 2.5% (*w*/*v*) iodoacetamide (IAA). For the second dimension of separation, the strips were applied directly to a 12% SDS-polyacrylamide gel. Immediately after 2D-DIGE was performed, the gels were scanned with a Pharos FX™ Plus Molecular Imager scanner using excitation/emission filters of 532/580 nm for Cy3 and 633/670 nm for Cy5 to generate multiplexed DIGE image files. Statistical and quantitative analyses of the changes in the spots on the images were performed using Progenesis SameSpots software v4.1 (Nonlinear Dynamics). Spots that were only present in the FABP4-His-incubated cells were selected for further identification. Lastly, the gel was stained with a PlusOne Silver Staining Kit (GE Healthcare), following the manufacturer's recommendations.

Protein spots were selected and digested with trypsin [38]. Briefly, the gel pieces were de-stained by three washes in 25 mM NH_4HCO_3, with vortexing, for 15 min and one wash in 100% CH_3CN. The gel pieces were dried and then swelled by incubation with digestion buffer (50 mM NH_4HCO_3 and 12.5 ng/mL of autolysis-stabilized trypsin) (Promega, Madison, WI, USA) at 37 °C for 16 h. The peptides were extracted sequentially in 0.1% trifluoroacetic acid (TFA) and 0.1% TFA in 50% CH_3CN and vacuum-dried. Lastly, the peptides were washed using Zip-tip C18 columns (Millipore), and the elutions were recovered with a 50% CH_3CN and 0.1% TFA solution.

The peptides were spotted onto an AnchorChip (Bruker, Bremen, Germany) target using α-cyano-4-hydroxy-cinnamic acid as a matrix. The peptides were then analyzed with a MALDI TOF/TOF (Ultraflextreme, Bruker Daltonics) instrument operated in the positive ion mode. The analyzed mass range was 700–3500 Da, with ion suppression of up to 600 Da. MS and MS/MS analyses were performed automatically. For the MS analysis, 1500 single-shot spectra were accumulated by recording 50-shot spectra at 10 random positions using fixed laser attenuation. The selection of precursor ions for MS/MS was performed using an Autoexecute workflow with FlexControl software v3.4 (Bruker Daltonics). For the MS/MS analysis, 100 single-shot spectra were recorded for precursors, and 3000 single-shot spectra were recorded for the fragment ion spectra. The peptide and tandem mass spectra were searched using MASCOT (Matrix Science Inc., MA) against the Swiss-Prot database (released on 03/2013), focusing on human taxonomy. The search parameters were as follows: MS accuracy = 75 ppm, MS/MS accuracy = 0.7 Da, two missed cleavages by trypsin allowed, carbamidomethyl of cysteine as a fixed modification and oxidation of methionine as a variable modification.

2.10. GeLC-MS/MS analysis

The concentrations of plasma membrane proteins from HUVECs that were incubated with or without FABP4-His were measured by the Bradford assay, using BSA as a standard. SDS-PAGE analysis was carried out with 5 μg of these samples, which were run on a 12% acrylamide minigel. After completion of the run, the gel was visualized by colloidal Coomassie Blue stain (Invitrogen Life Technologies). Following visualization of the gels, each lane was completely cut out in ten pieces using a scalpel and placed into a 0.5-mL Eppendorf tube for in-gel digestion.

The protein bands were excised from the gel and shrunk by dehydration in acetonitrile, which was then removed. The resulting spots were dried in a vacuum centrifuge. The gel pieces were covered with 10 mM dithiothreitol (DTT), and the proteins were reduced for 1 h at 56 °C. After cooling to RT, the DTT solution was replaced with approximately the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3. After 45 min of incubation at an ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 100 mM NH_4HCO_3 for 10 min, dehydrated by the addition of acetonitrile, swelled by rehydration in 100 mM NH_4HCO_3, and shrunk again by addition of the same volume of acetonitrile. The liquid phase was removed, and the gel pieces were completely dried in a vacuum centrifuge. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/mL of autolysis-stabilized trypsin (Promega) in an ice-cold bath. After 45 min, the supernatant was removed and replaced with the same buffer without trypsin to keep the gel pieces wet during enzymatic cleavage (37 °C, overnight). The peptides were extracted by one change of 20 mM NH_4HCO_3 and three changes of 5% formic acid in 50% acetonitrile (20 min for each change) at RT and dried.

Analysis was performed with an Esquire HCT ion trap mass spectrometer (Bruker) coupled to a nano-HPLC system (Proxeon, Denmark). The samples were first concentrated on a 300-μm i.d. 1-mm PepMap nanotrapping column and then loaded onto a 75-μm i.d. 15-cm PepMap nanoseparation column (LC Packings, The Netherlands). The peptides were eluted with an acetonitrile gradient (gradient: 0–60% B in 60 min; B = 80% acetonitrile and 0.1% formic acid in water; flow rate = 300 nL/min) through a PicoTip emitter nano-spray needle (New Objective, Woburn, MA, USA) onto the nanospray ionization

source of the ion trap mass spectrometer. MS/MS fragmentation (1.9 s, m/z 100–2800) was performed for two of the most intense ions, as determined from a 1.2-s MS survey scan (m/z 310–1500), using a dynamic exclusion time of 1.2 min for precursor selection. An automated optimization of the MS/MS fragmentation amplitude starting from 0.60 V was used. The proteins were identified by searching the UniProt-SwissProt 57.9 human database using Mascot (Matrix Science, London, UK). The MS/MS spectra were searched with a precursor mass tolerance of 1.5 Da, fragment tolerance of 0.5 Da, trypsin specificity with a maximum of two missed cleavages allowed, carbamidomethylation as a fixed modification, and methionine oxidation as a variable modification. The positive identification criterion was an individual Mascot score for each peptide MS/MS spectrum that was higher than the corresponding homology threshold score.

2.11. Statistical analysis

The results are the average of 3 independent experiments, which were each performed in duplicate (presented as the mean ± SEM). GraphPad Prism was used for statistical analysis (version 5, San Diego, CA, USA), and Student's t-test was used to compare the means. Differences between the two groups were considered statistically significant at $p < 0.05$.

3. Results

3.1. Exogenous FABP4 is found in plasma membranes of HUVECs

We incubated HUVECs with or without FABP4-His for 5 min, 15 min, 30 min, 1 h, 2 h and 4 h, and the plasma membrane protein extracts were analyzed. An anti-FABP4 immunoblot revealed a 2.2-fold increase in FABP4 levels when the cells were incubated with FABP4-His for 5 min compared with the cells incubated without FABP4-His ($p < 0.05$). When cells were incubated without exogenous FABP4, the levels remained constant throughout the 4 h of the experiment (Fig. 1A).

HUVECs were incubated with or without exogenous FABP4 for 5 min, and double-immunofluorescence staining with anti-FABP4 and anti-CD31 (a HUVEC plasma membrane protein marker) without permeabilization was performed. Confocal images revealed colocalization of FABP4 and CD31 (Fig. 1B). Immunofluorescence images showed an increased presence of FABP4 in cells that were incubated with exogenous FABP4 compared to those that were not (1.3-fold increase, $p < 0.05$) (Fig. 1C).

After knocking down FABP4 expression with siRNA, we repeated the immunofluorescence experiments without permeabilization and observed an increase in the amount of FABP4 in HUVECs incubated with exogenous FABP4 (2.3-fold increase, $p < 0.05$) (Fig. 1C).

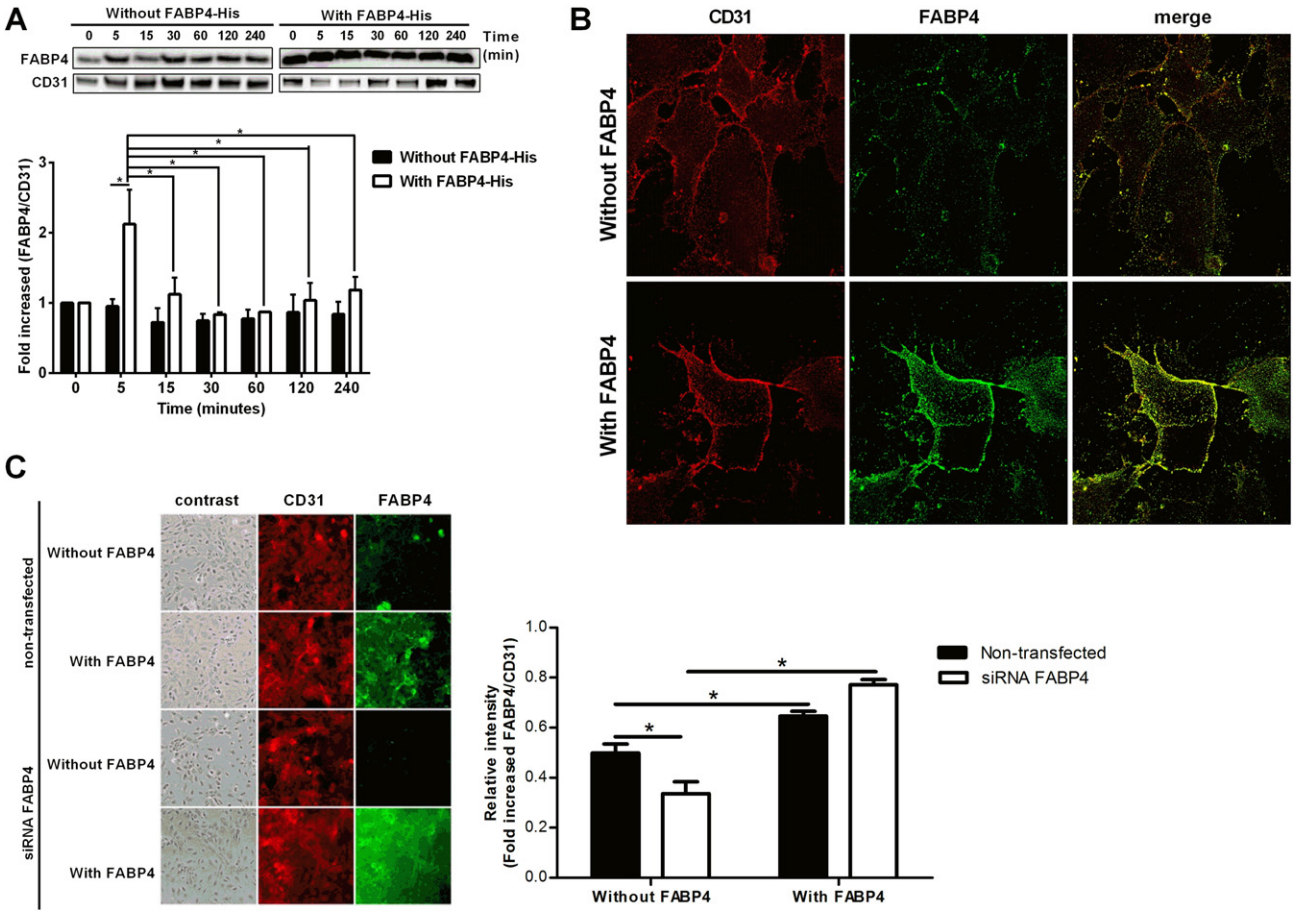

Fig. 1. The presence of exogenous FABP4 in the plasma membranes of HUVECs. (A) FABP4 and CD31 western blots of plasma membrane protein lysates from HUVECs that were incubated with or without exogenous FABP4 (100 ng/mL) for different times (0–4 h). The bar graph represents the quantitation of FABP4 expression levels in the plasma membrane. (B) Confocal images from double immunofluorescence staining for CD31 (Alexa Fluor® 532 dye) and FABP4 (Alexa Fluor® 488 dye), without plasma membrane permeabilization. The cells were incubated with or without exogenous FABP4 for 5 min. (C) Double immunofluorescence staining for CD31 (Alexa Fluor® 532 dye) and FABP4 (Alexa Fluor® 488 dye) was performed without membrane permeabilization in both non-transfected and FABP4-siRNA-transfected HUVECs that were incubated with or without exogenous FABP4 for 5 min. The quantification of FABP4 was performed after normalization for CD31 levels. The results are presented as the mean ± SEM and represent the average of 3 independent experiments (*$p < 0.05$).

3.2. Exogenous FABP4 interacts specifically with plasma membrane proteins

We performed a ligand-screening assay using plasma membrane proteins from HUVECs to determine whether the proteins present in the extracts were bound to FABP4. We analyzed increasing protein concentrations (from 0 to 60,000 pg/mL) of plasma membrane proteins from HUVECs incubated with or without exogenous FABP4 (100 ng/mL) for 5 min. We observed that when cells were incubated with exogenous FABP4, the amount of FABP4 protein detected by fluorescence increased in a concentration-dependent manner (Fig. 2A). This increase was presumably due to the presence of exogenous FABP4 in the plasma membrane. However, when cells were incubated without exogenous FABP4, we observed a concentration-dependent reduction in fluorescence, indicating the presence of FABP4 ligands in the plasma membranes of the HUVECs (Fig. 2A).

Next, HUVECs were incubated with or without FABP4-His (100 ng/mL) for 5 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-Tag purification and western blotting. An anti-FABP4 western blot showed no bands for the cells incubated without FABP4-His, and 4 different bands (approximately 16, 33, 77 and 108 kDa) were detected for the cells incubated with FABP4-His (Fig. 2B and Supp. Fig. 1S). An anti-6X His-tag western blot also showed the 4-band profile (Fig. 2C). HUVECs that were incubated with high levels of tag-free FABP4 (100 ng/mL) and low levels of FABP4-His (10 ng/mL) showed only 16- and 33-kDa bands (the 33-kDa band was a FABP4 homodimer [39]), indicating that the bands at 77 and 108 kDa represented specific complexes formed by exogenous FABP4 and its putative receptor (Fig. 2B and Suppl. Fig. 1S).

We incubated HUVEC plasma membrane proteins in the presence or absence of FABP4-His inside cobalt columns to confirm complex formation. Anti-FABP4 and anti-6X His-tag immunoblots showed 16-, 33-, 77- and 108-kDa bands, confirming the existence of these complexes (Fig. 2D).

We next knocked down FABP4 expression in HUVECs (Figs. 1C, 2E) and incubated the cells with or without FABP4-His (100 ng/mL) for 5 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-tag purification and western blotting. An anti-FABP4 western blot (Fig. 2F) showed 4 different bands (approximately 15, 33, 77 and 108 kDa) in the FABP4-deficient cells incubated with FABP4.

HUVECs were incubated with or without FABP4-His (100 ng/mL) for 5 min, 10 min or 15 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-Tag purification and western blotting to determine whether the interaction between FABP4 and its putative receptor was stable or transient. We observed that the complex formation peaked at 5 min (Fig. 3A); the amount of the complex observed at the plasma membrane began to decrease after 5 min.

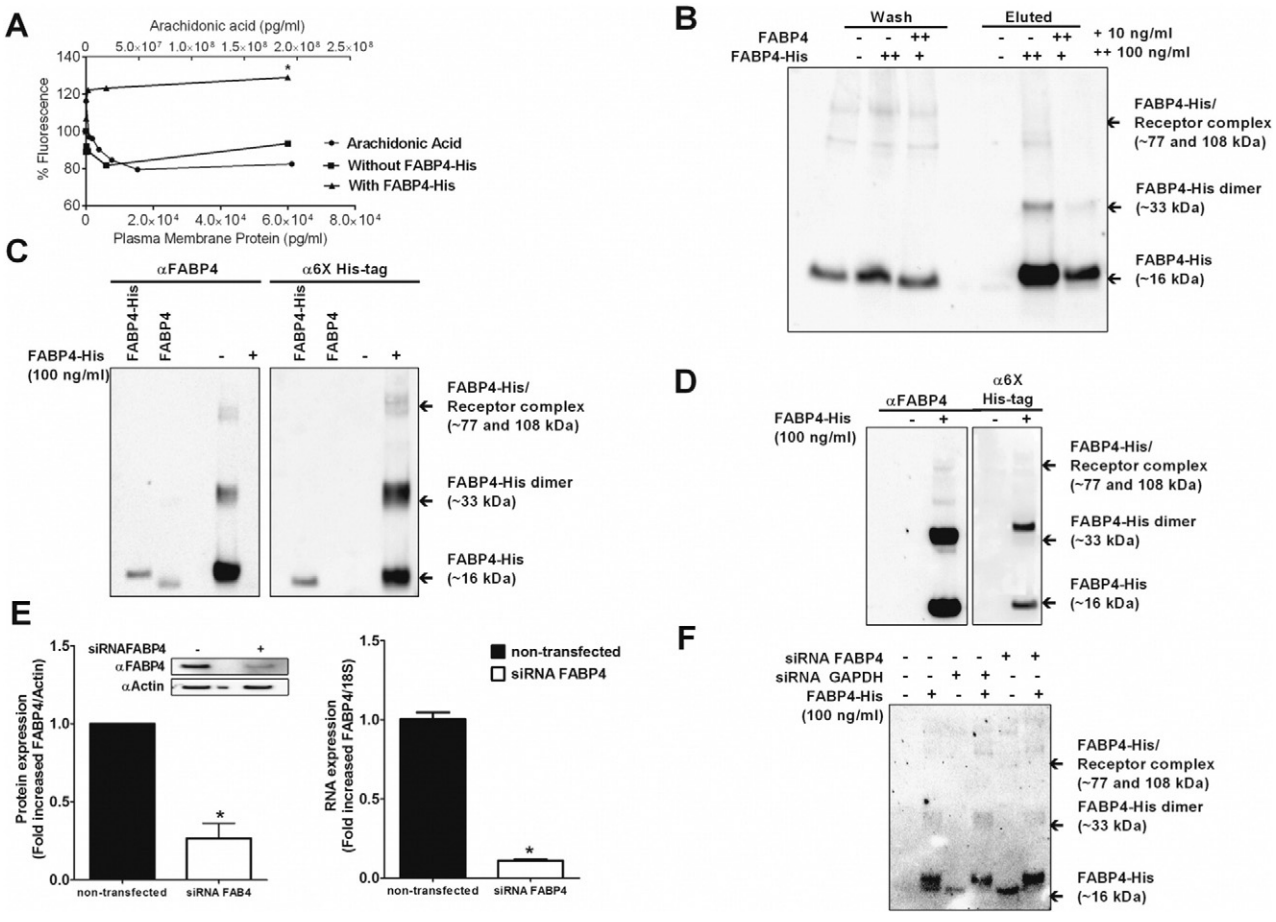

Fig. 2. Specific interactions between exogenous FABP4 and plasma membrane proteins from HUVECs. (A) Displacement curves of plasma membrane proteins from HUVECs incubated with or without FABP4-His (100 ng/mL); arachidonic acid (1.28 mM) was included as a positive control. (B) Western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His and after poly-His-tag purification (eluted and wash fractions), using antibodies against FABP4. (C) Western blot of FABP4-His recombinant protein, FABP4 recombinant protein and plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His and after poly-His-tag purification, using antibodies against FABP4 and the 6X His-tag. (D) Western blot of plasma membrane proteins incubated in cobalt resin columns with or without FABP4-His and visualized with anti-FABP4 and anti-6X His-tag antibodies. (E) FABP4 expression after transfecting HUVECs with siRNA FABP4 as determined by western blot analysis and quantitative real-time PCR. (F) FABP4 western blot of plasma membrane protein lysates from non-transfected and FABP4- and GAPDH-siRNA-transfected HUVECs that were incubated with or without FABP4-His and subjected to poly-His-tag purification. The results are presented as the mean ± SEM and represent the average of 3 independent experiments ($^*p < 0.05$).

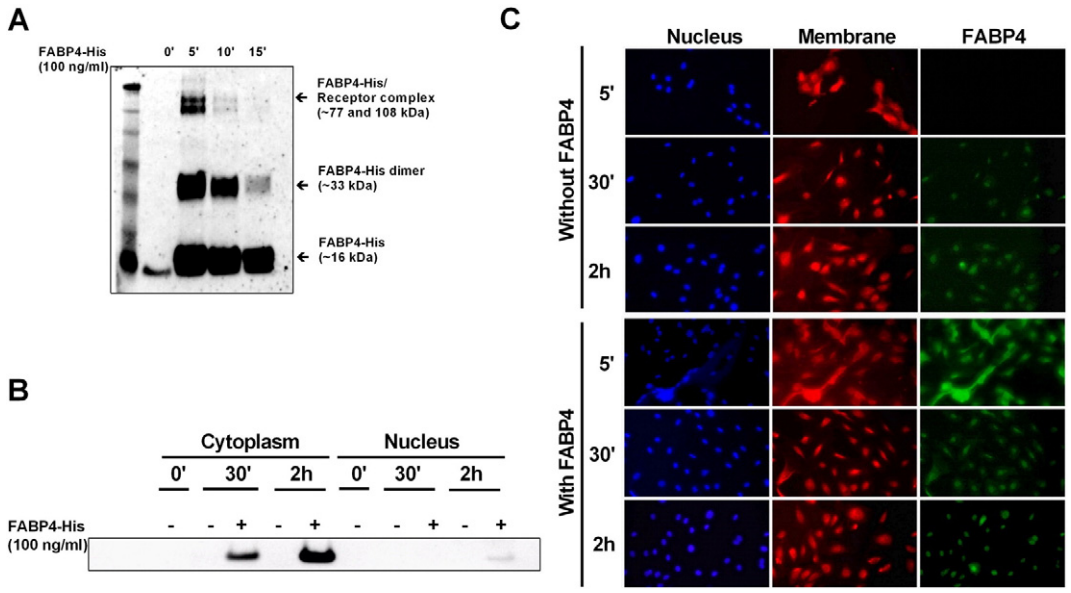

Fig. 3. Exogenous FABP4 is internalized by HUVECs. (A) FABP4 western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4 (100 ng/mL) for different times (0–15 min) and poly-His-tag purification. (B) Western blot, using antibodies against FABP4, of cytoplasmic and nuclear protein lysates from HUVECs that were incubated with or without FABP4-His (100 ng/mL) for different times (0–2 h) and subjected to poly-His tag purification. (C) Triple immunofluorescence staining of the nucleus (DAPI), membrane (CellMask) and FABP4 (Alexa Fluor® 488 dye) was performed on permeabilized HUVECs that were incubated with or without exogenous FABP4 for three different periods of time (5 min, 30 min or 2 h).

To investigate whether exogenous FABP4-His crosses the plasma membrane to enter the cytoplasm and nucleus in HUVECs, we incubated cells with or without FABP4-His for different times (0 min, 30 min or 2 h) and extracted the cytoplasmic and nuclear proteins. Following poly-His-tag purification, immunoblot analysis revealed the presence of FABP4 in the cytoplasm after incubation with exogenous FABP4 for 30 min or 2 h and the presence of FABP4 in the nucleus after incubation with exogenous FABP4 for 2 h (Fig. 3B). After permeabilizing the cells, triple fluorescence staining with anti-FABP4, CellMask and DAPI was performed on HUVECs that had been incubated with or without exogenous FABP4 for 5 min, 30 min or 2 h. In cells that were incubated with exogenous FABP4, the FABP4 immunofluorescence (green) changed from being associated with the plasma membrane (5 min) to being associated with the nucleus (2 h), which indicated that FABP4 migrated from the plasma membrane to the nucleus (Fig. 3C). Conversely, in cells that were not incubated with exogenous FABP4, the same immunofluorescence profile did not appear (Fig. 3C). These data were corroborated by western blot (Fig. 3B).

3.3. Fatty acids are required for the formation of FABP4 protein complexes

HUVECs were incubated with or without FABP4-His (100 ng/mL) in the presence or absence of the inhibitor BMS309403 (10 μM) for 5 min. This incubation was followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-tag purification and western blotting. Fig. 4 indicates weak decreases in the formation of the 108 and 77 kDa complexes, suggesting that the binding of fatty acids to FABP4 was necessary for these interactions.

3.4. Identification of cytokeratin 1 as the binding protein

Two-dimensional gel electrophoresis was performed with plasma membrane extracts from HUVECs that were incubated with or without FABP4-His followed by formaldehyde cross-linking, plasma membrane protein extraction and poly-His-tag purification (Fig. 5A). The samples were labeled with different CyDye fluorochromes (Cy5 for proteins

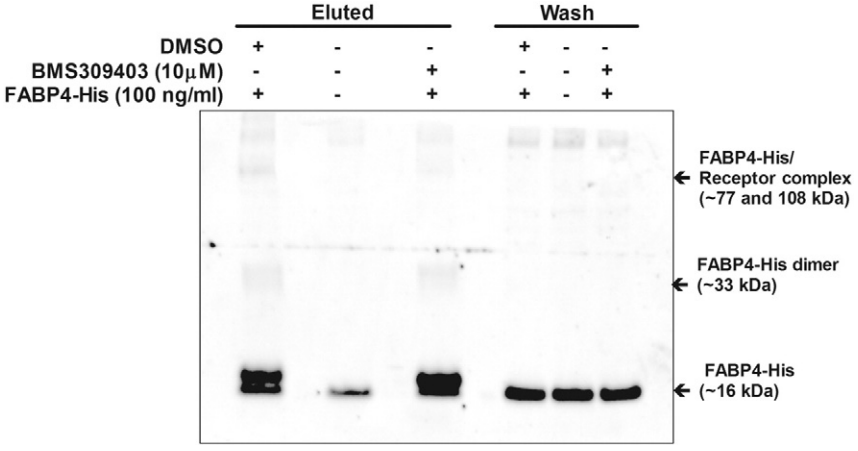

Fig. 4. BMS309403 decreases exogenous FABP4 complex formation. FABP4 western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His (100 ng/mL), with or without BMS309403 (10 μM), and following poly-His tag purification (eluted and wash fractions).

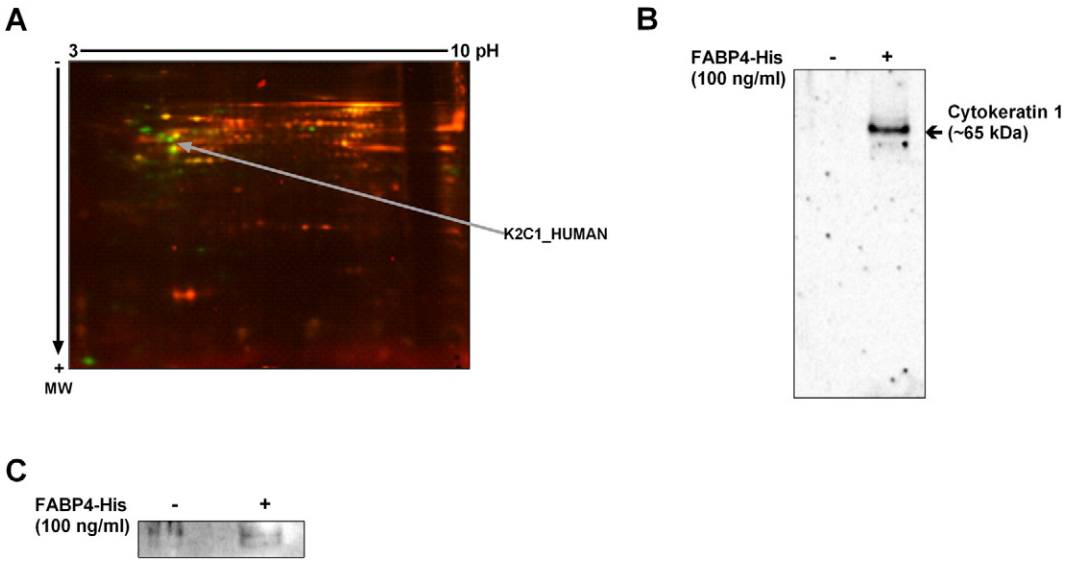

Fig. 5. Identification of CK1 as a FABP4 binding protein. (A) Representative overlapping 2D-GEL expression maps of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His (100 ng/mL) and following labeling with fluorescent dyes: Cy3 (green) corresponds to incubation with FABP4, and Cy5 (red) corresponds to incubation without FABP4. This image is representative of 1 of 3 analyzed gels. (B) CK1 western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His and poly-His tag purification. (C) Western blot of plasma membrane proteins that were incubated in cobalt resin columns with or without FABP4-His and visualized with antibodies against CK1.

from HUVECs incubated without FABP4-His and Cy3 for proteins from HUVECs incubated with FABP4-His). A total of 98 spots were detected, 82 of which were detected in the samples incubated both with and without exogenous FABP4 and 16 that were only present in the sample incubated with exogenous FABP4. These 16 spots were excised, trypsin-digested and analyzed by MALDI-TOF/TOF. The mass spectrometry results showed a compatible binding profile between FABP4 and cytokeratins, specifically human CK1 (P04264 (K2C1_HUMAN)). This identification was confirmed by another proteomic strategy, GeLC-MS/MS analysis, for which protein samples were fractioned by performing 1D-gel electrophoresis prior to nano-LC ESI. Each lane was cut into 10 small pieces, in-gel digestion was performed, and the peptides were analyzed with a nano-LC ESI q-TOF mass spectrometer. Lastly, definitive validation was achieved by western blot using an anti-CK1 antibody (Fig. 5B). The immunoblot revealed three bands of approximately 108, 77 and 65 kDa. The 108- and 77-kDa complexes were formed by FABP4 and CK1, while the 65-kDa band represented CK1 released from the complexes in the western blot.

3.5. Exogenous FABP4 forms protein complexes in HAECs, HCASMCs, HepG2 cells and THP-1 cells

HAECs, HCASMCs, HepG2 cells and THP-1 cells were incubated with or without FABP4-His (100 ng/mL) for 5 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-tag purification and western blotting. The anti-FABP4 immunoblot (Fig. 6A) showed the 4-band profile (15, 33, 77 and 108 kDa) for these cells, indicating that FABP4 formed protein complexes with plasma membrane proteins. The anti-CK1 immunoblot (Fig. 6B) also indicated that these protein complexes were formed by FABP4 and CK1.

4. Discussion

The major finding of this study is that exogenous FABP4 is able to interact with plasma membrane proteins, forming specific protein complexes with CK1 in HUVECs. We demonstrated that exogenous FABP4 is localized in the plasma membrane in specific protein complexes in HUVECs and that fatty acids are required to form FABP4 protein complexes.

Previous studies have postulated that FABP4 is a plasmatic biomarker of metabolic syndrome, T2DM and atherosclerotic disease [24,26–28]. In this study, interactions between exogenous FABP4 and the plasma membranes of HUVECs were shown, supporting the hypothesis that circulating FABP4 is not simply a biomarker but that it may have a causal effect by interacting with peripheral cells. In addition, we observed that these interactions occurred through specific protein complexes, even in FABP4-deficient HUVECs.

We observed that exogenous FABP4 could be internalized into HUVECs, passing through the cell membrane into the cytoplasm after 30 min and into the nucleus after 2 h. It is known that CK1 is an endothelial receptor that aids in the internalization of myeloperoxidase [40], so it is possible that CK1 also aids in the internalization of FABP4. However, it is not known whether this internalization occurs due to the interaction between FABP4 and CK1 or whether free FABP4 (15 kDa) can pass through the membrane into the cytoplasm; both

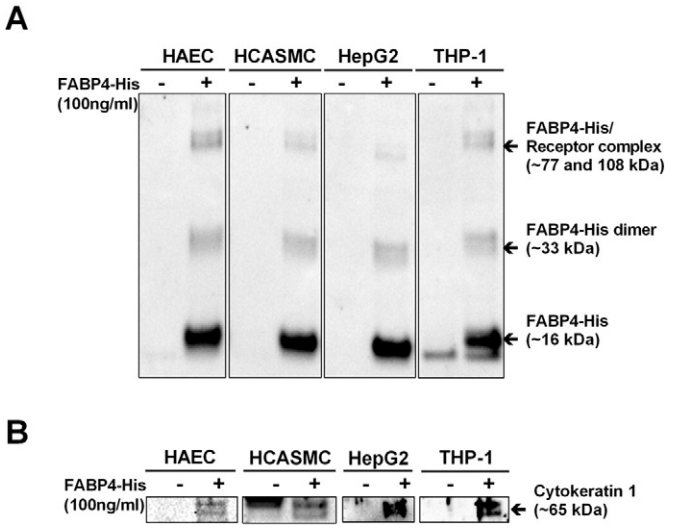

Fig. 6. Specific protein interactions of exogenous FABP4 and CK1 in HAECs, HCASMCs, HepG2 cells and THP-1 cells. FABP4 (A) and CK1 (B) western blots of plasma membrane protein lysates from HAECs, HCASMCs, HepG2 cells and THP-1 cells after incubation with or without FABP4-His (100 ng/mL) and following poly-His tag purification.

means of internalization are possible. We do know, however, that FABP4 and CK1 interact only transiently in the plasma membrane given that levels of the protein complex decreased after 5 min. In support of our results, the amount of intracellular FABP4 has been observed to increase following FABP4 incubation compared with non-treated cells [33]. It has also been shown that exogenous FABP4 can be taken up into cells [41].

We observed that administration of a FABP4 inhibitor (BMS309403) decreased complex formation between exogenous FABP4 and CK1. Therefore, fatty acids play an important role in FABP4 functionality, decreasing its interaction with plasma membrane proteins from HUVECs. BMS309403 is a biphenyl azole inhibitor designed to target FABP4. This compound competitively binds within the FABP4 fatty acid-binding pocket and inhibits the binding of FABP4 to endogenous free fatty acids [42,43]. Mechanistically, BMS309403 inhibits lipid accumulation, cholesterol efflux and inflammatory responses in macrophages, suppresses fatty acid uptake in adipocytes in a FABP4-dependent manner and stimulates glucose uptake through selective activation of the AMPK signaling pathway [43,44]. It is possible that the protection against atherosclerosis and T2DM provided by BMS309403 is due in part to a decrease in FABP4 complex formation.

This study used two proteomics strategies, both of which identified CK1 as the FABP4-binding protein in the plasma membranes of HUVECs. First, using MALDI TOF/TOF mass spectrometry, we analyzed plasma membrane protein lysates from HUVECs incubated with or without FABP4-His after cross-linking and purification with cobalt resin. The MALDI TOF/TOF profiles revealed that CK1 was the putative FABP4-binding protein. We repeated the procedure but used GeLC-MS/MS for the analysis, which also showed that CK1 was the putative receptor. CK1 was again confirmed to be the FABP4 receptor by immunoblot.

Cytokeratins are known to be part of a family of intermediate filament proteins that participate in cytoskeletal assembly [45]. CK1 is a member of the basic-neutral subfamily of cytokeratins [46]. In endothelial cells, CK1 appears to play a role as an anchor or receptor for various active molecules [45]. CK1 was also shown to colocalize with uPAR to form a multiprotein receptor complex for high-molecular-weight kininogen binding at the cell surface [47,48]. Furthermore, it was demonstrated that endothelial oxidative stress increases CK1 cell-surface protein expression and its ability the bind proteins (mannose-binding lectin) [49]. In addition, CK1 has been determined to be involved in vascular biology by regulating nitric oxide (NO) production [45], acting as a scaffolding protein for the assembly of the vasoregulatory plasma kallikrein–kinin system [40]. The kallikrein–kinin system was first recognized as a plasma and tissue proteolytic system that is responsible for the liberation of bradykinin, a vasoactive, proinflammatory mediator, resulting in NO synthesis and liberation. Tissue plasminogen activator release, superoxide formation, and prostacyclin formation are also induced by bradykinin [45]. It is known that oxidative stress and circulating FABP4 levels are increased in metabolic diseases, such as T2DM and obesity [19–25,50,51]. In our study, we hypothesized that oxidative stress could be involved in FABP4-CK1 interaction. Previous results from our group showed that exogenous FABP4 has a functional role in endothelial cells, inducing endothelial dysfunction. Exogenous FABP4 inhibits the activation of the insulin-signaling pathway, resulting in decreased eNOS activation and NO production [33]. Therefore, the FABP4-CK1 binding could decrease NO production and induce endothelial dysfunction. We also demonstrated that FABP4 inhibition decreases complex formation, likely by ameliorating endothelial dysfunction.

Furthermore, CK1 can be phosphorylated, suggesting that kininogen binding may induce intracellular signaling [45]. Exogenous FABP4 is also able to activate intracellular pathways (MAPK and Akt-dependent pathways) [33,34]; thus, it is possible that this activation also occurs through the FABP4-CK1 interaction.

We showed that this interaction did not occur in HUVECs only but that it also took place in other cell types, such as HAECs, HCASMCs, HepG2 cells and THP-1 cells. These results suggest that high levels of FABP4 in the circulation are not simply a clinical manifestation of cardiometabolic risk but are also a causative factor for cardiovascular disease. It is especially striking that exogenous FABP4 was able to form a protein complex with CK1 in HepG2 cells because these cells do not express FABP4, supporting the importance of the interaction of circulating FABP4 with peripheral tissues.

In conclusion, we demonstrated that FABP4 is not just a biomarker of metabolic diseases. Circulating FABP4 interacts with plasma membrane proteins, specifically CK1. Furthermore, FABP4 is able to cross the plasma membrane into the cytoplasm and reach the nucleus, although its role in this location is not fully known. Pharmacological inhibition of FABP4 can modulate its mechanism of action, decreasing complex formation and therefore reducing activity associated with the FABP4-CK1 interaction. Therefore, FABP4 represents a potential therapeutic target for the prevention of cardiovascular diseases that are associated with obesity and T2DM. Thus, the findings from this study and others suggest that FABP4 inhibition should be explored as a potential therapeutic strategy for treating atherosclerosis and reducing cardiovascular risk.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.09.002.

Transparency document

The Transparency document associated with this article can be found, in online version.

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References

[1] A.W. Zimmerman, J.H. Veerkamp, New insights into the structure and function of fatty acid-binding proteins, Cell. Mol. Life Sci. 59 (2002) 1096–1116.
[2] A.V. Hertzel, D.A. Bernlohr, The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function, Trends Endocrinol. Metab. 11 (2000) 175–180.
[3] D.A. Bernlohr, M.A. Simpson, A.V. Hertzel, L.J. Banaszak, Intracellular lipid-binding proteins and their genes, Annu. Rev. Nutr. 17 (1997) 277–303.
[4] N.H. Haunerland, F. Spener, Fatty acid-binding proteins—insights from genetic manipulations, Prog. Lipid Res. 43 (2004) 328–349.
[5] L. Makowski, G.S. Hotamisligil, The role of fatty acid binding proteins in metabolic syndrome and atherosclerosis, Curr. Opin. Lipidol. 16 (2005) 543–548.
[6] C. Wolfrum, Cytoplasmic fatty acid binding protein sensing fatty acids for peroxisome proliferator activated receptor activation, Cell. Mol. Life Sci. 64 (2007) 2465–2476.
[7] A.E. Jenkins-Kruchten, A. Bennaars-Eiden, J.R. Ross, W.J. Shen, F.B. Kraemer, D.A. Bernlohr, Fatty acid-binding protein-hormone-sensitive lipase interaction. Fatty acid dependence on binding, J. Biol. Chem. 278 (2003) 47636–47643.
[8] B.R. Thompson, A.M. Mazurkiewicz-Munoz, J. Suttles, C. Carter-Su, D.A. Bernlohr, Interaction of adipocyte fatty acid-binding protein (AFABP) and JAK2: AFABP/aP2 as a regulator of JAK2 signaling, J. Biol. Chem. 284 (2009) 13473–13480.
[9] H. Elmasri, C. Karaaslan, Y. Teper, E. Ghelfi, M. Weng, T.A. Ince, H. Kozakewich, J. Bischoff, S. Cataltepe, Fatty acid binding protein 4 is a target of VEGF and a regulator of cell proliferation in endothelial cells, FASEB J. 23 (2009) 3865–3873.
[10] M. Furuhashi, R. Fucho, C.Z. Gorgun, G. Tuncman, H. Cao, G.S. Hotamisligil, Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice, J. Clin. Invest. 118 (2008) 2640–2650.
[11] G.S. Hotamisligil, R.S. Johnson, R.J. Distel, R. Ellis, V.E. Papaioannou, B.M. Spiegelman, Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein, Science 274 (1996) 1377–1379.
[12] K. Maeda, H. Cao, K. Kono, C.Z. Gorgun, M. Furuhashi, K.T. Uysal, Q. Cao, G. Atsumi, H. Malone, B. Krishnan, Y. Minokoshi, B.B. Kahn, R.A. Parker, G.S. Hotamisligil, Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes, Cell Metab. 1 (2005) 107–119.
[13] L. Scheja, L. Makowski, K.T. Uysal, S.M. Wiesbrock, D.R. Shimshek, D.S. Meyers, M. Morgan, R.A. Parker, G.S. Hotamisligil, Altered insulin secretion associated with reduced lipolytic efficiency in aP2 −/− mice, Diabetes 48 (1999) 1987–1994.

[14] B.O. Shum, C.R. Mackay, C.Z. Gorgun, M.J. Frost, R.K. Kumar, G.S. Hotamisligil, M.S. Rolph, The adipocyte fatty acid-binding protein aP2 is required in allergic airway inflammation, J. Clin. Invest. 116 (2006) 2183–2192.
[15] M. Furuhashi, G. Tuncman, C.Z. Gorgun, L. Makowski, G. Atsumi, E. Vaillancourt, K. Kono, V.R. Babaev, S. Fazio, M.F. Linton, R. Sulsky, J.A. Robl, R.A. Parker, G.S. Hotamisligil, Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2, Nature 447 (2007) 959–965.
[16] I. Schlottmann, M. Ehrhart-Bornstein, M. Wabitsch, S.R. Bornstein, V. Lamounier-Zepter, Calcium-dependent release of adipocyte fatty acid binding protein from human adipocytes, Int. J. Obes. 38 (2014) 1221–1227.
[17] T. Mita, M. Furuhashi, S. Hiramitsu, J. Ishii, K. Hoshina, S. Ishimura, T. Fuseya, Y. Watanabe, M. Tanaka, K. Ohno, H. Akasaka, H. Ohnishi, H. Yoshida, S. Saitoh, K. Shimamoto, T. Miura, FABP4 is secreted from adipocytes by adenyl cyclase-PKA- and guanylyl cyclase-PKG-dependent lipolytic mechanisms, Obesity (Silver Spring) (2014)http://dx.doi.org/10.1002/oby.20954.
[18] M. Erikci Ertunc, J. Sikkeland, F. Fenaroli, G. Griffiths, M.P. Daniels, H. Cao, F. Saatcioglu, G.S. Hotamisligil, Secretion of fatty acid binding protein aP2 from adipocytes through a non-classical pathway in response to adipocyte lipase activity, J. Lipid Res. (2014) 423–434.
[19] A. Cabre, I. Lazaro, M. Cofan, E. Jarauta, N. Plana, A.L. Garcia-Otin, J.F. Ascaso, R. Ferre, F. Civeira, E. Ros, L. Masana, FABP4 plasma levels are increased in familial combined hyperlipidemia, J. Lipid Res. 51 (2010) 1173–1178.
[20] A. Cabre, I. Lazaro, J. Girona, J.M. Manzanares, F. Marimon, N. Plana, M. Heras, L. Masana, Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic patients, Atherosclerosis 195 (2007) e150–e158.
[21] A. Cabre, I. Lazaro, J. Girona, J.M. Manzanares, F. Marimon, N. Plana, M. Heras, L. Masana, Plasma fatty acid-binding protein 4 increases with renal dysfunction in type 2 diabetic patients without microalbuminuria, Clin. Chem. 54 (2008) 181–187.
[22] A. Cabre, I. Lazaro, J. Girona, J.M. Manzanares, F. Marimon, N. Plana, M. Heras, L. Masana, Plasma fatty acid binding protein 4 is associated with atherogenic dyslipidemia in diabetes, J. Lipid Res. 49 (2008) 1746–1751.
[23] B. Coll, A. Cabre, C. Alonso-Villaverde, I. Lazaro, G. Aragones, S. Parra, J. Girona, L. Masana, The fatty acid binding protein-4 (FABP4) is a strong biomarker of metabolic syndrome and lipodystrophy in HIV-infected patients, Atherosclerosis 199 (2008) 147–153.
[24] D. Stejskal, M. Karpisek, Adipocyte fatty acid binding protein in a caucasian population: a new marker of metabolic syndrome? Eur. J. Clin. Investig. 36 (2006) 621–625.
[25] A. Xu, Y. Wang, J.Y. Xu, D. Stejskal, S. Tam, J. Zhang, N.M. Wat, W.K. Wong, K.S. Lam, Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome, Clin. Chem. 52 (2006) 405–413.
[26] A.W. Tso, A. Xu, P.C. Sham, N.M. Wat, Y. Wang, C.H. Fong, B.M. Cheung, E.D. Janus, K.S. Lam, Serum adipocyte fatty acid binding protein as a new biomarker predicting the development of type 2 diabetes: a 10-year prospective study in a Chinese cohort, Diabetes Care 30 (2007) 2667–2672.
[27] A. Xu, A.W. Tso, B.M. Cheung, Y. Wang, N.M. Wat, C.H. Fong, D.C. Yeung, E.D. Janus, P.C. Sham, K.S. Lam, Circulating adipocyte-fatty acid binding protein levels predict the development of the metabolic syndrome: a 5-year prospective study, Circulation 115 (2007) 1537–1543.
[28] D.C. Yeung, A. Xu, C.W. Cheung, N.M. Wat, M.H. Yau, C.H. Fong, M.T. Chau, K.S. Lam, Serum adipocyte fatty acid-binding protein levels were independently associated with carotid atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 1796–1802.
[29] G. Aragones, R. Ferre, I. Lazaro, A. Cabre, N. Plana, J. Merino, M. Heras, J. Girona, L. Masana, Fatty acid-binding protein 4 is associated with endothelial dysfunction in patients with type 2 diabetes, Atherosclerosis 213 (2010) 329–331.
[30] H.E. Agardh, L. Folkersen, J. Ekstrand, D. Marcus, J. Swedenborg, U. Hedin, A. Gabrielsen, G. Paulsson-Berne, Expression of fatty acid-binding protein 4/aP2 is correlated with plaque instability in carotid atherosclerosis, J. Intern. Med. 269 (2011) 200–210.
[31] W. Peeters, D.P. de Kleijn, A. Vink, S. van de Weg, A.H. Schoneveld, S.K. Sze, P.J. van der Spek, J.P. de Vries, F.L. Moll, G. Pasterkamp, Adipocyte fatty acid binding protein in atherosclerotic plaques is associated with local vulnerability and is predictive for the occurrence of adverse cardiovascular events, Eur. Heart J. 32 (2011) 1758–1768.
[32] V. Lamounier-Zepter, C. Look, J. Alvarez, T. Christ, U. Ravens, W.H. Schunck, M. Ehrhart-Bornstein, S.R. Bornstein, I. Morano, Adipocyte fatty acid-binding protein suppresses cardiomyocyte contraction: a new link between obesity and heart disease, Circ. Res. 105 (2009) 326–334.
[33] G. Aragones, P. Saavedra, M. Heras, A. Cabre, J. Girona, L. Masana, Fatty acid-binding protein 4 impairs the insulin-dependent nitric oxide pathway in vascular endothelial cells, Cardiovasc. Diabetol. 11 (2012) 72.
[34] J. Girona, R. Rosales, N. Plana, P. Saavedra, L. Masana, J.C. Vallve, FABP4 induces vascular smooth muscle cell proliferation and migration through a MAPK-dependent pathway, PLoS One 8 (2013), e81914.
[35] R. Glass, A. Loesch, P. Bodin, G. Burnstock, P2X4 and P2X6 receptors associate with VE-cadherin in human endothelial cells, Cell. Mol. Life Sci. 59 (2002) 870–881.
[36] C. Klockenbusch, J. Kast, Optimization of formaldehyde cross-linking for protein interaction analysis of non-tagged integrin beta1, J. Biomed. Biotechnol. 2010 (2010) 927585.
[37] J. Vasilescu, X. Guo, J. Kast, Identification of protein–protein interactions using in vivo cross-linking and mass spectrometry, Proteomics 4 (2004) 3845–3854.
[38] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.
[39] R.E. Gillilan, S.D. Ayers, N. Noy, Structural basis for activation of fatty acid-binding protein 4, J. Mol. Biol. 372 (2007) 1246–1260.
[40] J.M. Astern, W.F. Pendergraft 3rd, R.J. Falk, J.C. Jennette, A.H. Schmaier, F. Mahdi, G.A. Preston, Myeloperoxidase interacts with endothelial cell-surface cytokeratin 1 and modulates bradykinin production by the plasma kallikrein–kinin system, Am. J. Pathol. 171 (2007) 349–360.
[41] H. Uehara, T. Takahashi, M. Oha, H. Ogawa, K. Izumi, Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression, Int. J. Cancer (2014) 2558–2568.
[42] X. Hui, H. Li, Z. Zhou, K.S. Lam, Y. Xiao, D. Wu, K. Ding, Y. Wang, P.M. Vanhoutte, A. Xu, Adipocyte fatty acid-binding protein modulates inflammatory responses in macrophages through a positive feedback loop involving c-Jun NH2-terminal kinases and activator protein-1, J. Biol. Chem. 285 (2010) 10273–10280.
[43] W. Lin, X. Huang, L. Zhang, D. Chen, D. Wang, Q. Peng, L. Xu, J. Li, X. Liu, K. Li, K. Ding, S. Jin, J. Li, D. Wu, BMS309403 stimulates glucose uptake in myotubes through activation of AMP-activated protein kinase, PLoS One 7 (2012), e44570.
[44] M.Y. Lee, H. Li, Y. Xiao, Z. Zhou, A. Xu, P.M. Vanhoutte, Chronic administration of BMS309403 improves endothelial function in apolipoprotein E-deficient mice and in cultured human endothelial cells, Br. J. Pharmacol. 162 (2011) 1564–1576.
[45] A.A. Hasan, T. Zisman, A.H. Schmaier, Identification of cytokeratin 1 as a binding protein and presentation receptor for kininogens on endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 3615–3620.
[46] Z. Shariat-Madar, A.H. Schmaier, Kininogen–cytokeratin 1 interactions in endothelial cell biology, Trends Cardiovasc. Med. 9 (1999) 238–244.
[47] F. Mahdi, Z. Shariat-Madar, R.F. Todd 3rd, C.D. Figueroa, A.H. Schmaier, Expression and colocalization of cytokeratin 1 and urokinase plasminogen activator receptor on endothelial cells, Blood 97 (2001) 2342–2350.
[48] F. Mahdi, Z.S. Madar, C.D. Figueroa, A.H. Schmaier, Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes, Blood 99 (2002) 3585–3596.
[49] C.D. Collard, M.C. Montalto, W.R. Reenstra, J.A. Buras, G.L. Stahl, Endothelial oxidative stress activates the lectin complement pathway: role of cytokeratin 1, Am. J. Pathol. 159 (2001) 1045–1054.
[50] L. Polimeni, M. Del Ben, F. Baratta, L. Perri, F. Albanese, D. Pastori, F. Violi, F. Angelico, Oxidative stress: new insights on the association of non-alcoholic fatty liver disease and atherosclerosis, World J. Hepatol. 7 (2015) 1325–1336.
[51] S. Tangvarasittichai, Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus, World J. Diabetes 6 (2015) 456–480.