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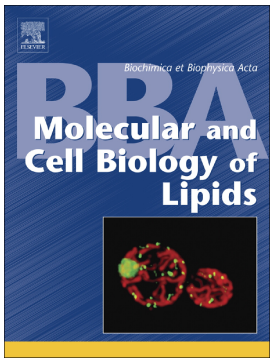

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PII: S1388-1981(18)30367-6
DOI: https://doi.org/10.1016/j.bbalip.2018.11.011
Reference: BBAMCB 58390

To appear in: *BBA - Molecular and Cell Biology of Lipids*

Received date: 18 April 2018
Revised date: 14 November 2018
Accepted date: 30 November 2018

Please cite this article as: N. Martínez-Micaelo, R. Rodríguez-Calvo, S. Guaita-Esteruelas, M. Heras, J. Girona, L. Masana , Extracellular FABP4 uptake by endothelial cells is dependent on cytokeratin 1 expression. Bbamcb (2018), https://doi.org/10.1016/j.bbalip.2018.11.011

Extracellular FABP4 uptake by endothelial cells is dependent on cytokeratin 1 expression

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Running title: Cytokeratin 1 mediates FABP4 cellular uptake in endothelial cells.

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Abstract

Aims: The aim of this study is to determine the physical and functional interplay between fatty acid-binding protein 4 (FABP4) and its membrane receptor-like candidate protein, cytokeratin 1 (CK1), and to determine the effect of hindering CK1-mediated FABP4 cellular uptake on non-disturbed or metabolically stressed endothelial cells.

Methods: We monitored the direct interaction between FABP4 and CK1 using surface plasmon resonance, and the effects of blocking exogenous FABP4 (eFABP4) cellular uptake were determined by using specific siRNA to knock down the expression of CK1 in human umbilical vein endothelial cells (HUVECs). The expression and nuclear translocation of transcription factors involved in oxidative stress (NRF2) and inflammation (p65 subunit of NF-κB transcription factor) were determined by Western blotting analysis.

Results: Our data showed that FABP4 and CK1 bind to each other and that the putative FABP4 binding domain would be within the ^{151}GIQEVTINQSLLQPLNVEID^{170} CK1 sequence. We determined that in non-disturbed or metabolically stressed endothelial cells, eFABP4 regulates the cellular response to oxidative stress. In addition, we also found that in the presence of palmitate, eFABP4 increases the pro-inflammatory effects induced by palmitate *per se*, probably due to an increase in the transport of palmitate inside cells, suggesting that these FABP4-mediated pro-oxidative and pro-inflammatory effects are dependent on CK1 expression.

Conclusions: We demonstrated that CK1 facilitates eFABP4 cellular uptake in endothelial cells. Therefore, the CK1-targeted inhibition of exogenous FABP4 cellular uptake might be a potential therapeutic strategy to protect endothelial cells against FABP4-induced activation of inflammation and oxidative stress.

Keywords: FABP4, CK1, endothelial dysfunction, inflammation

Highlights:

- CK1 and FABP4 interact physically and functionally in endothelial cells.
- CK1 expression affects the cellular uptake of eFABP4 and the effects mediated by eFABP4-transported fatty acids, including the activation of inflammation and oxidative stress processes.
- The interaction between CK1 and FABP4 in the membrane of endothelial cells would provide a potential target for the prevention of eFABP4-mediated endothelial dysfunction and therefore a new therapeutic strategy for preventing cardiovascular diseases associated with obesity and diabetes.

1. Introduction

Obesity-induced excess of adipose tissue storage capacity results in adipocyte dysfunction, leading to a lipolysis-mediated increased concentration of fatty acids (FA) in plasma [1], and induces the secretion of a wide range of adipose tissue-derived factors, including the fatty acid-binding protein 4 (FABP4) [2–4].

FABP4 is a 14-kDa cytosolic protein expressed in adipocytes [5], macrophages [6], and endothelial cells [7], and increased circulating expression of FABP4 is linked with insulin resistance, type 2 diabetes and cardiovascular diseases [8]. FABP4 reversibly binds to a single saturated or unsaturated long-chain FA with high affinity, transporting it intracellularly to the different cellular compartments [9]. Under obesogenic conditions, FABP4 is actively released by adipocytes and acts as an adipokine [10], that is, a biologically active protein that can be taken up by different cell types, such as the endothelial cells [11]. In addition to their role as an energy source, FA are also molecular signals. Therefore, FABP4 might play a key role in the regulation of lipid-mediated processes including the activation of inflammation and oxidative stress processes.

Circulating FABP4 can modulate endothelial cell function. In fact, previous studies of our group have revealed that exogenous FABP4 (eFABP4) has a functional role in endothelial cells [12]. Moreover, eFABP4 inhibits the activation of the insulin-signaling pathway, resulting in decreased eNOS activation and NO production inducing endothelial dysfunction [13], an event considered as the first process in the pathogenesis of atherosclerosis. However, the molecular mechanisms that regulate eFABP4 cellular uptake are still poorly understood. Consistently, in another study, we showed that cytokeratin 1 (CK1) and eFABP4 interact transiently in the membrane of endothelial cells [14], even though we did not evaluate the role of CK1 in the endocytosis of eFABP4 and its functional implication.

Cytokeratins belong to a large family of intermediate filament proteins with cytoskeletal assembly properties [15]. CK1, a basic-neutral type of cytokeratin, is found on the surface of endothelial cells, where it acts as a receptor-like protein [16]. In endothelial cells, CK1 has been associated with urokinase plasminogen activator (uPAR) receptor as a multiprotein receptor for high-molecular-weight kininogen (HK), prekallikrein and 2-chain urokinase plasminogen [17]. CK1 was also identified as a protein that facilitates endothelial internalization of myeloperoxidase [18] and c-reactive protein (CRP) [19].

The results presented in this study demonstrate the key role of CK1 as a receptor-like protein that facilitates the cellular uptake of eFABP4 in non-disturbed or metabolically stressed endothelial

cells, identifying blockade of eFABP4 internalization as a novel therapeutic strategy to decrease FABP4-mediated activation of inflammation and oxidative stress.

2. Materials and methods

2.1. Cell culture and reagents

The human umbilical vein endothelial cells (HUVECs) were cultured in Medium 200 supplemented with 2% low serum growth supplement (LSGS) and 1% gentamicin/amphotericin (GIBCO, Oregon, OR, USA) at 37°C in a humidified incubator with 5% CO_2. In the current study, HUVECs were used at passage 3.

Human recombinant FABP4 (100 ng/ml) was purchased from BioVendor (Heidelberg, Germany), and human recombinant polyhistidine-tag FABP4 (FABP4-His) was from Enzo Life Sciences (San Diego, CA, USA). The CK1-siRNA, FABP4-siRNA and the control-siRNA were acquired from Dharmacon (Lafayette, CO, USA). The anti-Ck1 and anti-FABP4 were purchased in BioVendor, and the anti-p65 and anti-NRF2 were purchased in Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin and IgG-HRP were purchased from Santa Cruz Biotechnology and Dako (Glostrup, Denmark), respectively. Sodium palmitate, sodium oleate, acid eicosapentanoic (EPA) and acid linoleic were purchased in NU-Chek Prep (Elysian, MN, USA). CK1 fragments were synthesized by Pepmic (Suzhou, Jiangsu, China). High-molecular-weight kininogen (HK) were purchased in Sigma-Aldrich (Darmstadt, Germany).

2.2. Surface plasmon resonance (SPR)

The interaction analyses between immobilized FABP4 and the CK1 fragments were performed on a Biacore 3000 instrument. A capture sensor surface was prepared by covalent immobilization of recombinant human FABP4 (Biovendor) to the dextran-coated (CM5) chip surface at 25ºC, by the standard primary amine coupling reaction. FABP4 in coupling buffer (10 mM sodium acetate buffer, pH 5.0) was injected over the activated chip surface until a resonance unit (RU) signal approximately 1.000 RU was reached. Ethanolamine was used to block unwanted carboxy groups after linkage. Equilibration of the baseline was completed by a continuous flow of PBS-T buffer, pH 7.4, through the chip for 1 h. An ethanolamine blocked channel was used as blank for background subtraction of non-specific signal. The activated coupled chip surfaces were then washed and treated with 50 mM NaOH to remove uncoupled residual proteins. All Biacore data were collected at 25ºC with PBS-T (pH 7.4) as running buffer at a constant flow of 20 $\mu l \cdot min^{-1}$. All of the sensorgrams were processed by using the automatic correction for nonspecific bulk refractive index effects.

The FABP4 (PDB reference: 4NNS) and CK1 peptide (manually generated from amino acid sequences) models were generated using PyMOL Molecular Graphic System, Version 2.0.6, Schrödinger, LLC.

2.3. Small interfering RNA (siRNA) transfection

Transfection of HUVECs with siRNA was performed with Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's protocol.

2.4. Membrane extract preparation

To obtain membrane extracts, HUVECs were incubated with ice-cold buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM Ca^{2+}, 1% Triton-X-100, 1% NP40, and protease inhibitors). After incubation, cell lysates were centrifuged, and the supernatant was collected and stored at -80°C.

2.5. Cytoplasm and nuclear extracts preparation

To obtain cytoplasmic and nuclear proteins, extracted cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in Buffer A (10 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCL, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol (DDT), protease and phosphatase inhibitors). Subsequently, cells were centrifuged, and the supernatant (cytoplasmic fraction) was collected and stored at -80°C. The nuclear pellet was washed once with PBS and resuspended in cold Buffer B (20 mM HEPES, 25% glycerol, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease and phosphatase inhibitors). The nuclear extract was obtained by centrifugation, and the supernatant, i.e., the nuclear fraction, was removed and stored at -80°C. The protein concentrations of the cytoplasmic and nuclear fractions were quantified with a Bradford assay using BSA as the standard.

2.6. Western blotting analysis

Membrane, cytoplasmic and nuclear proteins were electrophoresed on SDS-PAGE gels, and separated proteins were transferred onto a PVDF membrane. The blots were blocked and incubated overnight at 4°C with the appropriate primary antibody. After washes, the blots were incubated with a horseradish peroxidase secondary antibody, developed with ECL reagent (GE Healthcare, Buckinghamshire, UK) and exposed using the Amersham Imager 6000 (GE Healthcare). A semi-quantitative analysis of the proteins was performed using ImageQuant TL software.

2.7. Immunofluorescence staining of FABP4

Cells were washed, fixed in 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.5% Triton X-100. After incubation with blocking buffer, the cells were incubated with FABP4

primary antibody overnight at 4ºC and were then incubated with Alexa-Fluor 488 anti-goat antibody. Immunofluorescence images were acquired with the Olympus IX71 inverted microscope, processed with Cell^F Software (Olympus, Shinjuku-ku, Tokyo, Japan).

2.8. Statistical analysis

Results are expressed as the mean ± SEM. Effects of the pretreatments were assessed using ANOVA. We used a Tukey's test to make pairwise comparisons. Differences were considered significant when the *p* values were <0.05. All calculations were performed using SPSS 17.0 software.

3. Results

3.1. FABP4 directly interacts with CK1

Using SPR technology, we monitored the direct interaction between the CK1 protein and recombinant FABP4 immobilized to the surface of a sensor chip. To map the CK1 region involved in this interaction, three peptides that spanned portions of the H1 subdomain in the head region of this protein were designed (Figure 1A and 1B). In this way and based on previous studies describing the interaction between CK1 and one of its best-characterized ligands in endothelial cells, the high molecular weight kininogen (HK), we choose the binding domain of kininogen to CK1 as the potential FABP4 binding domain. The first designed peptide, identified as P1, covering from 7 AA before to 5 AA after the entire sequence of the kininogen binding domain, and the two other synthesized peptides, identified as P2 and P3, sequentially covering this binding region, while the P2 peptide covered the first 22 amino acids, the P3 covered the last 20 amino acids. Nevertheless, due to problems related to the purification process, the synthesis of P1 was not feasible.

Therefore, the series of SPR experiments were carried out at various concentrations of P2 and P3 peptides. We observed that P3 exhibited a remarkable binding affinity to FABP4, as a concentration-dependent increase of SPR signal was generated after the injection of P3 in the SPR spectrum (Figure 1C and 1D). However, the P2 injection showed no affinity to FABP4 protein, as no increasing response was observed in the SPR spectrum (Figure 1E). Therefore, the putative FABP4 binding domain would be within the ^{151}GIQEVTINQSLLQPLNVEID^{170} protein sequence of CK1.

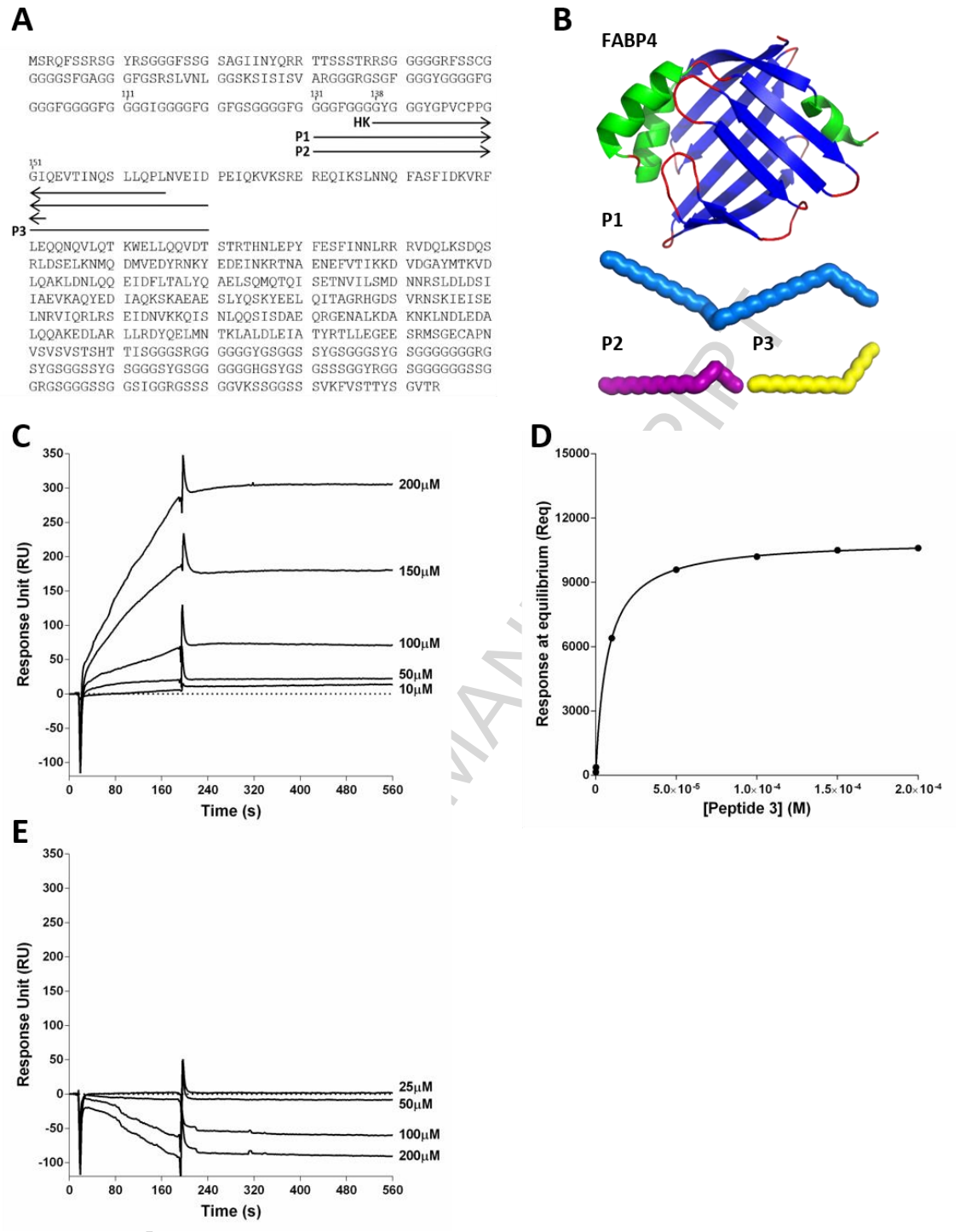

Figure 1. FABP4 and CK1 physically interact. (A) The amino acid sequence of cytokeratin 1, regions corresponding to the high-molecular-weight kininogen (HK), peptide 1 (P1), peptide 2 (P2) and peptide 3 (P3) were indicated. (B) 3D models of FABP4 and the 3 peptides derived from the CK1 sequence. (C) Interaction of FABP4 with CK1 peptide P3 was observed by surface plasmon resonance (SPR). The representative sensorgram show SPR response due to refractive index difference as a result of P3-FABP4 binding. (D) Equilibrium data analysis of CK1 peptide 3 binding to FABP4. Steady state RU vs. concentration of CK1 peptide 3 plot was obtained based on the 1:1 (Langmuir) binding fitting model. (E) No interaction was observed between the peptide P2 and the FABP4.

3.2. CK1 silencing decreases cellular uptake of eFABP4 in HUVECs

To test the CK1 and eFABP4 interplay at the cellular level, we determined how the silencing of CK1 expression could affect the presence of eFABP4 in the membrane of HUVECs. As we expected, after knocking down CK1 expression in cells with CK1-siRNA, the expression of this protein in the membrane of HUVECs decreased by more than half of the expression in cells transfected with the control-siRNA (Figure 2A and 2B).

Remarkably, we also observed that blocking CK1 expression significantly reduced the cellular uptake of eFABP4 was significantly reduced. Therefore, decreased FABP4 protein levels were observed in the membranes of cells transfected with CK1-siRNA compared with those transfected with the control-siRNA (Figure 2A and 2C). We also observed that the internalization of eFABP4 was diminished after CK1 silencing using histidine immunofluorescence staining (Figure 2D).

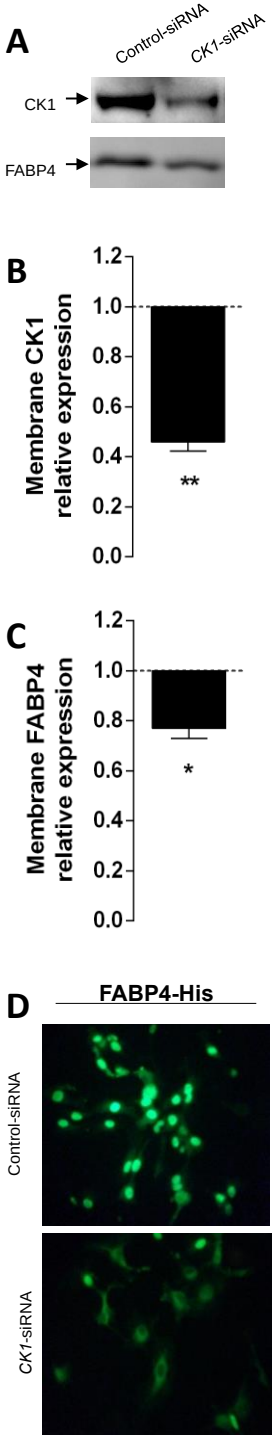

Figure 2. CK1 knockdown decreases the presence of FABP4 in the plasma membrane and the eFABP4 cellular uptake of HUVECs. (A) The presence of CK1 and FABP4 in the membrane of HUVECs were determined by Western blotting of the membrane lysates from HUVECs transfected with CK1- or control-siRNA and incubated with or without exogenous FABP4 (100 ng/mL) fro 5 min. (B, C) The effect of CK1 transfection on CK1 and FABP4 expression were quantified relative to the control group. (D) To evaluate the effect of CK1 knockdown in the uptake of eFABP4 HUVEC cells were transfected with CK1- or control-siRNA and incubated with FABP4-His (100 ng/mL) for 60 min. Exogenous FABP4 cellular uptake was visualized by immunofluorescence staining using an antibody against poly-His-tag (Alexa Fluor 488 dye) on permeabilized HUVECs. The results are presented as the mean ± S.E.M of 3 independent experiments. *P<0.05, **P<0.01, Student's t-test.

3.3. The knockdown of CK1 decreases eFABP4 cellular uptake and downstream signaling

HUVECs were transfected with CK1- or control-siRNA and then incubated with FABP4-His (100 ng/ml) for 60 min to study the role of CK1 on the intracellular migration of eFABP4. The internalization of eFABP4 was affected by the silencing of CK1. Specifically, using an anti-FABP4 immunoblot, we observed the decreased presence of eFABP4 in both the cytoplasm and the nucleus of the CK1-siRNA transfected cells (Figure 3A and 3D). We observed no effects of CK1 silencing on the pro-inflammatory subunit of NF-κB transcription factor in the cytoplasm or p65 nuclear translocation (Figure 3A and 3D-F). On the other hand, eFABP4 in CK1-silenced HUVECs showed decreased nuclear translocation and phosphorylation of NRF2 (Figure 3A, 3D and 3G-I).

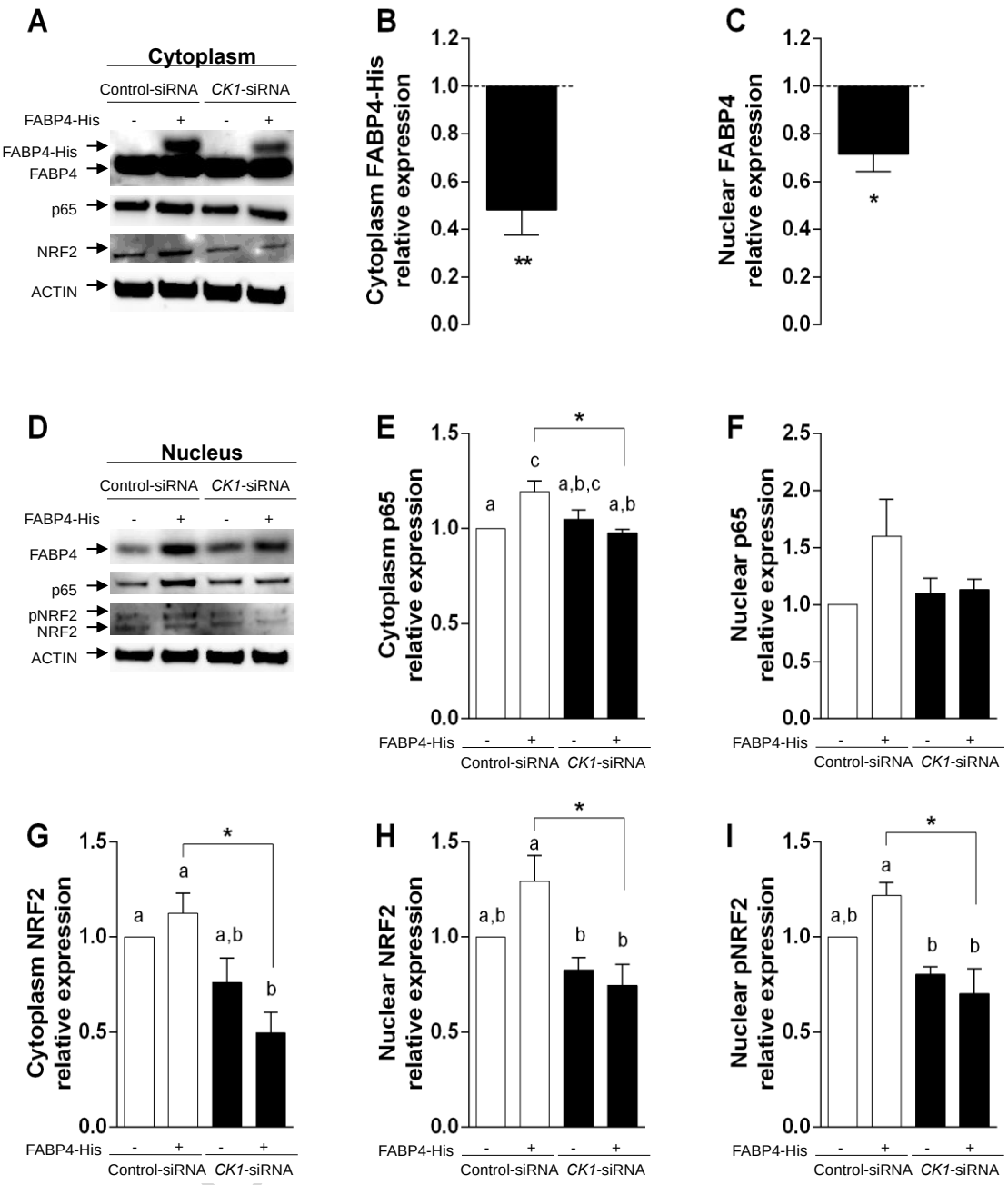

Figure 3. CK1 affects exogenous FABP4 cellular uptake and functional implications. HUVECs were transfected with CK1- or control-siRNA and incubated with or without FABP4-His (100 ng/mL) for 60 min. (A and D) Representative Western blotting analysis of FABP4, p65 and NRF2 expression in the cytoplasm and nucleus, respectively. Protein semi-quantifications in cytoplasm and nucleus are shown in FABP4 (B and C), p65 (E and F) and NRF2 (G-I). The results are presented as the mean ± S.E.M of 3 independent experiments. Different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$). *$P<0.05$ between cells transfected with CK1- and control-siRNA and incubated with FABP4-His, Student's t-test.

3.4. Exogenous FABP4 nuclear abundance is dependent on the fatty acid type

In a previous study, we determined that fatty acids would be necessary for the formation of FABP4-CK1 protein complexes in the membrane of endothelial cells [14]. Taking this into account, we studied the effect of pre-incubating FABP4 with different saturated and unsaturated fatty acids in its cellular uptake in HUVECs. Palmitate (16:0; saturated fatty acid), oleate (18:1; monounsaturated) fatty acid, eicosapentaenoic acid (20:5; n-3 polyunsaturated fatty acid) and linoleic acid (18:2; n-6 polyunsaturated fatty acid), at a final concentration of 200 μM, were pre-incubated in medium at 37ºC for 15 min in the presence or absence of 100 ng/ml of FABP4-His. After the pre-incubation period, medium was added to HUVECs for 60 min. An anti-FABP4 immunoblot revealed that the rate of eFABP4 nuclear migration depends on the type of fatty acid (Figure 4). We observed that only the HUVECs that were incubated with palmitate or oleate (without FABP4-His), increased the endogenous expression of FABP4 in cytoplasm. Interestingly, although no differences in the cytoplasmic location of eFABP4 due to the type of pre-incubated fatty acid were observed, only those cells that were treated with palmitate- or oleate-FABP4 increased the nuclear migration of exogenous eFABP4, and in fact, only the increase observed in the palmitate-FABP4 treated cells was significant.

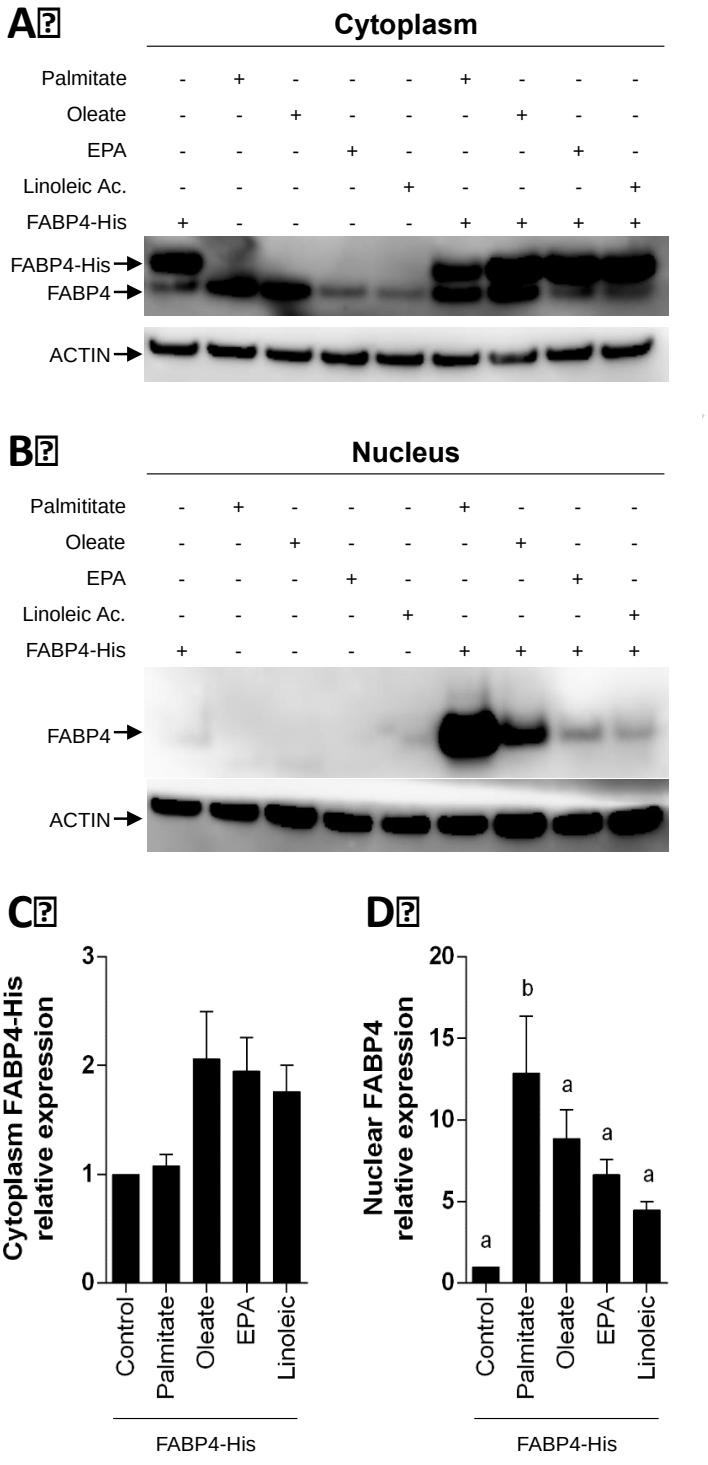

Figure 4. The nuclear abundance of FABP4 depends on the type of fatty acid. Medium supplemented with palmitate, oleate, eicosapentaenoic acid or linoleic acid in a final concentration of 200 μM, was pre-incubated in presence or absence of FABP4-His (100 ng/mL) for 15 min at 37ºC, then the medium was added to HUVECs for 60 min. (A and B) The FABP4 cellular uptake was determined using Western blotting analysis of the FABP4 expression in cytoplasm and nucleus, respectively. (C and D) Protein semi-quantifications of eFABP4 expression in cytoplasm and nucleus. The results represent the mean ± S.E.M of 3 independent experiments. Bars with different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$).

3.5. CK1 mediates palmitate-pre-incubated eFABP4 cellular uptake and its functional implications

We also investigated the role of CK1 in the internalization of eFABP4 in metabolically stressed endothelial cells. HUVECs were transfected with CK1- or control-siRNA and then treated with FABP4-His pre-incubated with palmitate. Using Western blotting analysis, we corroborated that the knockdown of CK1 also decreased the cytoplasmic and nuclear endocytosis of eFABP4 pre-incubated with palmitate (Figure 5A and 5D). Pre-incubating palmitate with eFABP4 increased the nuclear translocation of the p65 subunit of the NF-κB transcription factor compared with the cells treated with palmitate in the absence of eFABP4 (Figure 5A and 5D-5F). Furthermore, we also determined that eFABP4-palmitate effects were dependent on the presence of CK1. Thus, a sharp decrease in the nuclear translocation of the p65 was observed in CK1-silenced cells, as well as the presence of NRF2 in the cytoplasm and nucleus, and its activation was also decreased in CK1-silenced cells (Figure 5A, 5D and 5G-I).

Moreover, we analyzed the specificity of CK1-FABP4 interaction on the palmitate-mediated pro-inflammatory effect using different fatty acids delivers. To achieve this purpose, HUVECs were transfected with control-siRNA or with CK1- and FABP4-siRNA in order to avoid endogenous FABP4 effects, and after the transfection period, cells were treated with palmitate pre-incubated with recombinant FABP4, FABP5 or albumin (Figure 6). Using this approach, we corroborated that eFABP4 was taken up by HUVECs being detected both in the cytoplasm and in the nucleus and that CK1 was necessary for that. We also corroborated that pre-incubating palmitate with eFABP4, as well as FABP5 and albumin, increased the pro-inflammatory effect induced by palmitate *per se*. Furthermore, the knockdown of CK1 blocked the uptake of eFABP4 pre-incubated with palmitate resulting in a significant decrease in the nuclear translocation of p65, this inhibitory effect was slightly when CK1 and FABP4 silenced HUVECs were treated with palmitate pre-incubated FABP5 or albumin.

On the other hand, we also confirmed the key role of CK1 in the uptake of eFABP4 in HUVECs using HK to chemically-mediated blockade of CK1 instead of the siRNA-mediated knockdown model (Figure 7). HK-mediated blockade of CK1 reduced significantly the internalization of eFABP4 resulting in a slight decrease of p65 nuclear translocation.

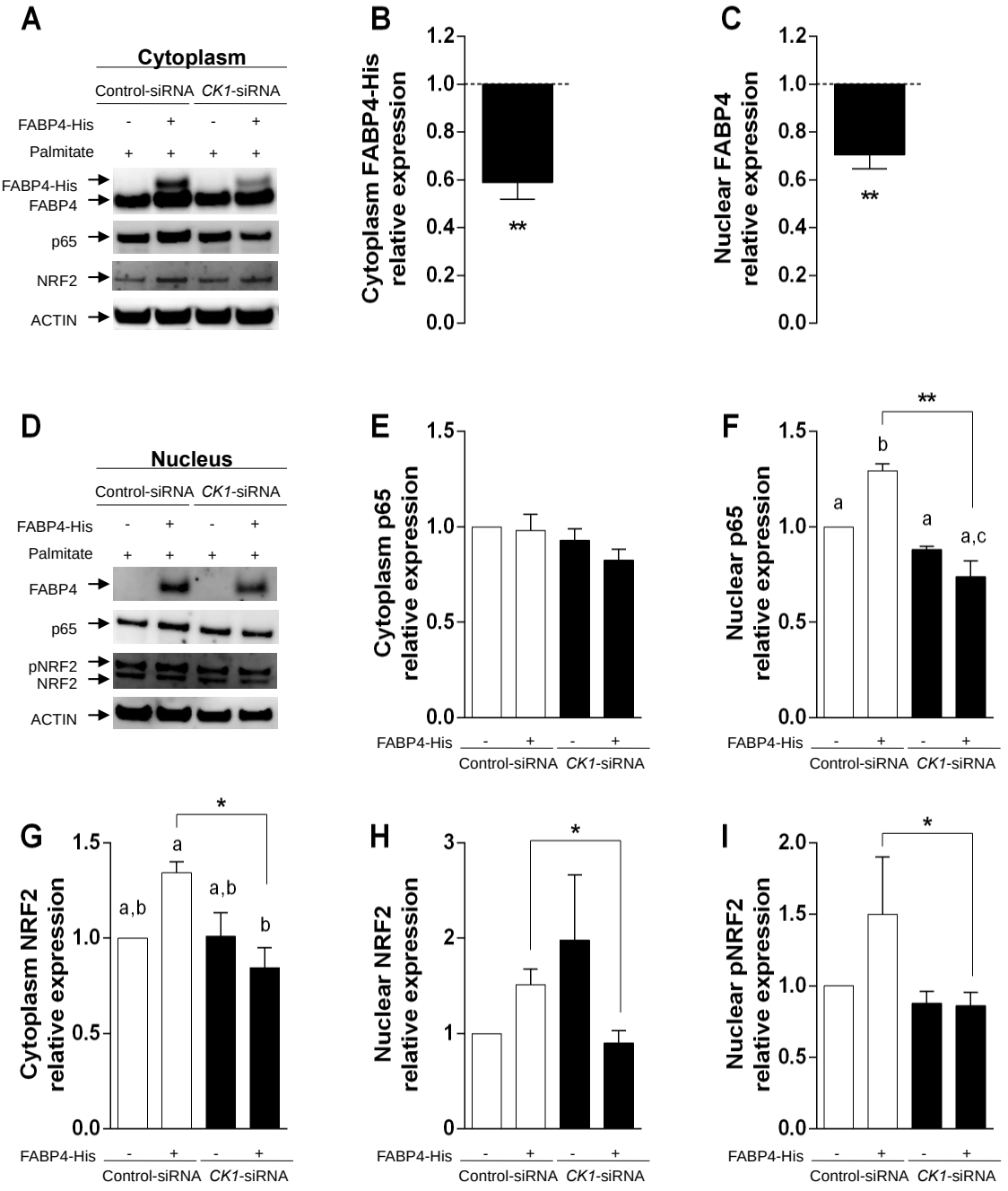

Figure 5. CK1 affects the cellular uptake and functions of palmitate-exposed FABP4. HUVECs were transfected with CK1- or control-siRNA and incubated with or without FABP4-His (100 ng/mL) incubated previously with palmitate (200 µM). (A and D) Representative Western blotting analysis of FABP4, p65 and NRF2 expression in the cytoplasm and nucleus, respectively. Protein semi-quantifications in cytoplasm and nucleus are shown for FABP4 (B and C), p65 (E and F) and NRF2 (G-I). The results are presented as the mean ± S.E.M of 3 independent experiments. Different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$). *P<0.05 between cells transfected with CK1- and control-siRNA and incubated with FABP4-His, Student's t-test.

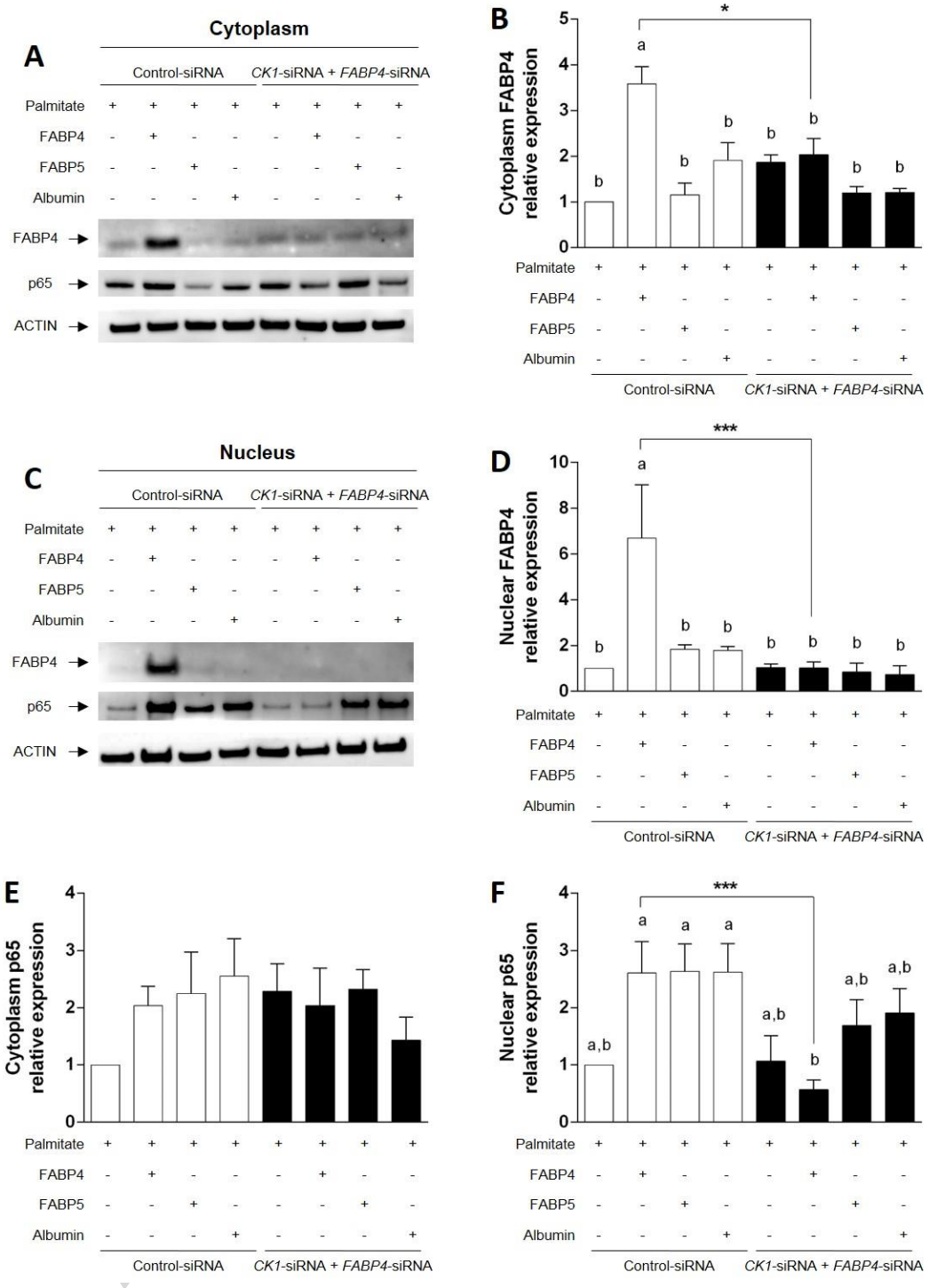

Figure 6. CK1 affects the cellular uptake of palmitate-exposed FABP4. HUVECs were transfected with CK1- and FABP4- or control-siRNA and incubated with or without FABP4 (100 ng/mL), FABP5 (100 ng/mL) or albumin, incubated previously with palmitate (200 μM). (A and C) Representative Western blotting analysis of FABP4 and p65 expression in the cytoplasm and nucleus, respectively. Protein semi-quantifications in cytoplasm and nucleus are shown for FABP4 (B and D) and p65 (E and F). The results are presented as the mean ± S.E.M of 3 independent experiments. Different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$). ***P<0.001, *P<0.05; between cells transfected with CK1- and FABP4- versus control-siRNA and incubated with FABP4, Student's t-test.

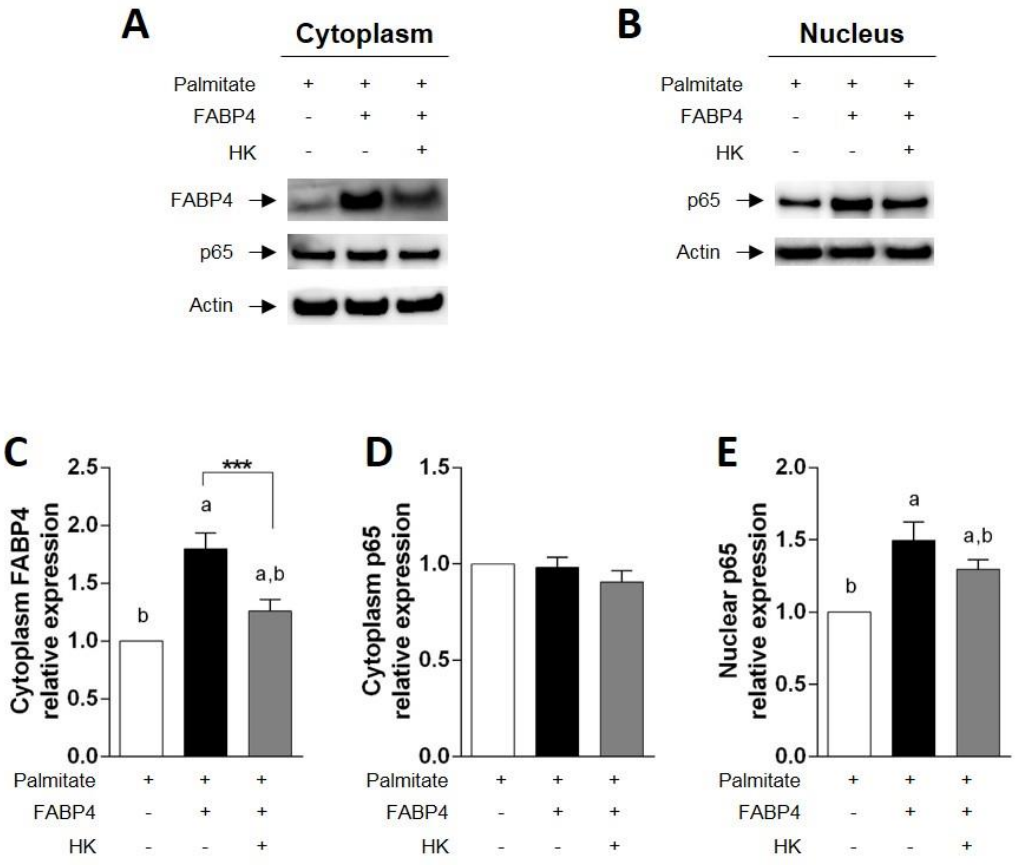

Figure 7. High molecular weight kininogen (HK)-mediated blockade of CK1 decrease exogenous FABP4 internalization. HUVECs were incubated with or without HK (1 ng/mL), and treated with palmitate (200 μM). (A and B) Representative Western blotting analysis of FABP4 and p65 expression in the cytoplasm and nucleus, respectively. Protein semi-quantifications in membrane are shown for FABP4 (C) in the cytoplasm and p65 (D and E) in the cytoplasm and nucleus, respectively. The results are presented as the mean ± S.E.M of 3 independent experiments. Different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$). ***P<0.001; between cells transfected with CK1- and FABP4- versus control-siRNA and incubated with FABP4, Student's t-test.

4. Discussion

The current study shows that CK1 and FABP4 interact physically and functionally in endothelial cells, leading to eFABP4 cellular uptake and eFABP4-mediated activation of inflammation and oxidative stress. Using SPR technology, the putative binding domain of FABP4 in CK1 was identified, and through the knockdown of CK1 expression in endothelial cells, the fundamental role of CK1 in the eFABP4 cellular uptake and internalization was corroborated. The present study also reported that in endothelial cells, CK1 expression affects not only the endocytosis of

eFABP4 to the cytoplasm and its nuclear translocation but also the effects mediated by the eFABP4-transported fatty acids, including the activation of inflammation and oxidative stress processes. In this way, targeting CK1 offers a new strategy to block eFABP4-mediated endothelial dysfunction.

Although FABP4 was originally described as an intracellular protein affecting lipid fluxes, metabolism and signaling within cells, in recent years it has become evident that FABP4 can be actively released into the circulation where it acts as a critical mediator of metabolism and inflammatory processes through hormonal-like functions including among others, the regulation of hepatic glucose production [20]. In fact, a large number of studies have shown that plasma levels of FABP4 are increased in obesity and type-2 diabetes [21], and that circulating FABP4 concentration correlated with clinical outcomes, such as body mass index, insulin resistance and dyslipidemia [22]. Therefore, considering that plasma-circulating FABP4 can be taken up by several cell types and that eFABP4 is able to modulate its function, independently if receptor cells endogenously express FABP4, identifies the understanding of FABP4 cellular uptake mechanisms as a promising basis to develop effective therapeutic strategies against the pathogenesis of metabolic and vascular diseases.

The vascular endothelium is a highly dynamic multifunctional organ that is critically involved in the regulation of vessel integrity, vascular growth and remodeling, tissue growth and metabolism, immune responses, cell adhesion, angiogenesis, hemostasis and vascular permeability [23]. Therefore, the proper functioning of the endothelium is important for the homeostasis of the body, and endothelial dysfunction is associated with several pathophysiological conditions, including atherosclerosis, hypertension, and diabetes [24]. Previous studies have reported that endothelial cells could internalize eFABP4 and that eFABP4 co-localized with CK1 in the membrane of endothelial cells forming specific protein complexes [14]. Considering this, the present study analyzes whether eFABP4 cellular uptake is dependent on CK1 interaction or if eFABP4 can pass through the membrane and be internalized in endothelial cells in a CK1-independent mechanism.

The results of the current study showed that FABP4 may interact with a CK1 derived peptide physically. Currently, the specific form of CK1 folding into a transmembrane protein in endothelial cells remains unclear. Therefore, to characterize the FABP4-CK1 binding, we based our work on previously determined structural characteristics. CK1 as an intermediate filament protein consists of 3 domains; a long central α-helical region, known as the rod domain (residues 180-489), flanked by two end domains, the head (amino-terminal, residues 2-179) and tail (carboxy-terminal, residues 490-644) [25,26]. The rod region has extensive regions of the coiled-coil structure; besides the amino- and carboxyl-end domains are highly flexible and characteristically have glycine-rich sequences forming glycine-loops through cell membranes

leading to the interaction with other membrane proteins. Thus, in order to map the putative FABP4 binding domain in the CK1 protein, we selected the H1 subdomain, which is the region that separates the rod region from the glycine-rich region in the head which terminates the penetration of the N-terminal region into cell membranes [17,27]. H1 is a highly conserved sequence in keratins, and previous studies reported this region as the binding domain of CK1 to HK, one of its best-characterized ligands in endothelial cells. Three peptides covering the selected region were designed, however, the synthesis of the peptide covering the entire region (named as P1) was not feasible, leading to determine the binding affinity between FABP4 and the peptides P2 and P3. Using this approach, we reported for the first time, that FABP4 and CK1 physically bind and that the FABP4 binding domain would be located among the ^{151}GIQEVTINQSLLQPLNVEID^{170} sequence corresponding to the P3 peptide. The specificity of this interaction was corroborated because no binding affinity was observed between FABP4 and the peptide P2. According to the primary structure analysis of Badowski *et al*. on the CK1 amino acid sequence [28], while the P2 peptide is covering a regulatory region containing several aromatic residues, such as phenylalanine (F) and tyrosine (Y) interspersed among glycine (G), cysteine (C), valine (V) and proline (P), forming a hydrophobic pocket, the P3 peptide is covering an aliphatic-rich region containing 50% of the residues with polar; glutamine (Q), threonine (T), asparagine (N) and serine (S), or charged characteristics; glutamate (E) and aspartate (D), properties. These differences would explain the different affinity shown by FABP4 towards one or the other peptide.

The role of CK1 as a receptor-like protein in the membrane of endothelial cells was previously described. Astern *et al.* [18] proposed that CK1 is part of a possible endothelial receptor complex formed together with the uPAR and the receptor for the globular head of complement 1q protein (gC1qR) and that this multiprotein receptor complex was the platform for the assembly and the activation of the vasoregulatory plasma kallikrein-kinin system. CK1 and uPAR, but not gC1qR, co-localize on the membrane of endothelial cells. Although uPAR binds to cell membrane constituents by a phosphatidylinositol linkage, CK1 has neither a phosphatidylinositol bond nor the structure of a transmembrane protein [17]. These results let us to hypothesize that CK1 and uPAR would form a receptor complex and their cell surface expression would be regulated by endocytosis and recycling. Therefore, the molecular mechanism through which CK1 and FABP4 interacts with the cell membrane of endothelial cells would be similar to the mechanism previously proposed for uPAR-mediated endocytosis; urokinase-plasminogen activator inhibitor 1 (uPA-PAI1) complexes bound to uPAR associated with low-density lipoprotein receptor-related protein 1 (LRP1) leading to clathrin-dependent endocytosis, lysosomal degradation of uPA andPAI1 and recycling of UPAR and LRP1 to the plasma membrane [29,30].

The second part of this study evaluated the role of CK1 in the eFABP4 cellular uptake in endothelial cells. By blocking CK1 expression using a specific siRNA, we corroborated that the presence of CK1 was fundamental for FABP4 cellular uptake and internalization in endothelial cells, thus decreased membrane expression of CK1 is associated with the decreased presence of exogenous FABP4 in the membrane, cytoplasm, and nucleus. We also evaluated the functional implication of the CK1-FABP4 interaction, and we observed that the inhibition of FABP4 internalization was related to decreased activation and nuclear translocation of the NRF2 transcription factor, although we did not observe effects on nuclear translocation of the p65 subunit of NF-κB in non-disturbed endothelial cells. Interestingly, previous studies reported that administration of a FABP4 inhibitor, the BMS309403, decreased the formation of the eFABP4-CK1 complex and that fatty acids would play an important role for FABP4 functionality [14]. Through the analysis of how different fatty acid types affect the internalization of eFABP4 in endothelial cells, we determined that eFABP4 nuclear translocation was fatty-acid-dependent. Our results corroborated the ligand-dependency of FABP4 nuclear translocation, so although FABP4 can bind numerous ligands, only specific compounds can activate its nuclear translocation [31]. Specifically, we found that although no differences attributed to the FA type were reported in eFABP4 expression in the cytoplasm, only eFABP4-transporting palmitate significantly increased eFABP4 nuclear translocation. It should be noted that elevated circulating concentrations of saturated free fatty acids, including palmitate, are implicated in obesity-associated inflammation and insulin resistance in endothelial cells [32]. Therefore, the next step was to assess whether knocking down CK1 or HK-mediated CK1 blocking would affect the endocytosis of palmitate-transporting eFABP4, in a metabolically stressed cell context. Using these approaches, we determined that eFABP4 cellular uptake is also mediated by CK1 in palmitate-stressed endothelial cells. Treating cells with eFABP4-transporting palmitate increased NRF2 expression in the cytoplasm and by blocking the expression of CK1, the phosphorylation and nuclear translocation of NRF2 were inhibited. Furthermore, we corroborated the role of FABP4 in the regulation of lipid-mediated processes, so when eFABP4 is pre-incubated with palmitate, the activation and nuclear translocation of the p65 subunit of NF-κB was increased compared with the non-FABP4 pre-incubated cells. In addition, by CK1-silencing, the nuclear translocation of p65 was significantly reduced. This pro-inflammatory effect of eFABP4 was not observed in the absence of palmitate. In unstressed cells, NRF2 and NF-κB transcription factors are maintained latent in cytoplasmic complexes, oxidative stress or pro-inflammatory stimuli, respectively, trigger the dissociation of these complexes leading to their nuclear import promoting the expression of downstream target genes. Both transcriptions factors are highly sensitive to their triggering stimulus, therefore, the decrease in the expression and activation of both transcription factors observed in CK1-silenced cells might be reflecting that cells are receiving less pro-oxidative and pro-inflammatory stimuli, that is, the less the cells are triggered, the less is the

response to the triggering stimulus. Therefore, eFABP4 regulates the cellular response to oxidative stress in non-disturbed or metabolically stressed endothelial cells. In addition, when cells are metabolically stressed using palmitate, eFABP4 enhances the pro-inflammatory effects induced by palmitate *per se*, possibly due to increased transport inside the cell. Moreover, these eFABP4-mediated effects were dependent on CK1 expression.

5. Conclusions

In conclusion, we demonstrated that CK1 is necessary for eFABP4 cellular uptake in endothelial cells and that the main effect of silencing CK1 is the blockade of eFABP4 cellular uptake, decreasing subsequently not only the effects mediated by eFABP4 *per se* but also the FABP4-transported lipid-mediated processes. Therefore, by targeting the CK1-FABP4 interaction in the membrane of endothelial cells, the endocytosis of eFABP4 is hindered in both, non-disturbed or metabolically stressed cells, offering a potential target for the prevention of eFABP4-mediated endothelial dysfunction and therefore a new therapeutic strategy for preventing cardiovascular diseases associated with obesity and diabetes. Further studies are necessary to completely characterize the eFABP4 cellular uptake and endocytosis mechanism as well as to study the regulation of other proteins involved in the cellular uptake of fatty acids, including CD36 and the fatty acid transport protein family (FATP), when CK1-FABP4 interaction is blocked.

Acknowledgements

This research was financially supported by a grant from ISCIII, Madrid, Spain (PI15/00627), Fondo Europeo de Desarrollo Regional (FEDER) and from the CIBER in Diabetes and Associated Metabolic Disorders (CB07/08/0028).

Conflicts of interest

None declared.

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