Free fatty acids mediates human umbilical vein endothelial cells inflammation through toll-like receptor-4

L. CHEN1,2, C.-X. YU1, B. SONG1, W. CAI3, C. LIU2, Q.-B. GUAN1

1Department of Endocrinology, Shandong Provincial Hospital Affiliated to Shandong University, Shandong Clinical Medical Center of Endocrinology and Metabolism, Institute of Endocrinology and Metabolism; Shandong Academy of Clinical Medicine, Jinan, Shandong China
2Department of Endocrinology, the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China
3Department of Gynecology and Obstetrics, the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China

Abstract. – OBJECTIVE: To investigate the role of Toll-like receptor-4 (TLR4) in the free fatty acids (FFAs) induced human umbilical vein endothelial cells (HUVECs) inflammation and to explore the underlying mechanisms.

MATERIALS AND METHODS: HUVECs and HEK293 cell lines were obtained from Shanghai Type Culture Collection. Cell counting kit-8 (CCK8) and flow cytometry (FCM) were performed to examine the cell viability and apoptosis rate of HUVECs induced by FFAs treatments with or without infection of toll-like receptor-4 interference (TLR4i) adenovirus. Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the inflammatory cytokines release. Quantitative polymerase chain reaction (qPCR) and Western Blot (WB) were used to test the molecular mechanisms of inflammation.

RESULTS: FFAs induced inflammatory responses in HUVECs via modulating the TLR4 receptor complex. TLR4i adenovirus interference increased cell viability and decreased cell apoptosis rate. FFAs treatments significantly increased the expressions of inflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), C-C motif chemokine ligand 5 (CCL5) and CXC chemokine ligand 10 (CXCL10), while TLR4i adenovirus interference significantly reduced these cytokines levels. TLR4-mediated myeloid differential protein-88 (MyD88) expression activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inhabiting kappa B kinase-beta (IKK-β). TLR4i adenovirus interference decreased the expressions of these genes at both mRNA level and protein level.

CONCLUSIONS: TLR4 mediates FFAs induced inflammatory responses in HUVECs. TLR4 interference in HUVECs significantly reduces the inflammatory cytokines expression, decreases the cell apoptosis rate and increases cell viability.

Key Words: TLR4, Free fatty acids, HUVECs, Inflammation, Apoptosis.

Introduction

Obesity is becoming a global health issue, especially in most industrialized countries. The epidemic of obesity is closely related with the increased occurrence of cardiovascular and metabolic diseases1. Moreover, the accumulated adipose tissue is also a main source of inflammation2,3. This explains the endurance of sub-health status in obesity. Studies4-7 have shown that obesity is associated with the prevalence of bone and joint diseases, especially arthritis. Other than the increased mechanical stress and pressure on joints caused by obesity, inflammation mediated by obesity is also one of the most important causative factors5. Studies have shown that rheumatic diseases can be regulated by cytokines secreted from adipocytes8. The level of free fatty acids (FFA) released from adipose tissue is a great marker of obesity in individuals. The elevated level of FFAs in the serum has been shown to be detrimental to various target organs, such as pancreatic islets, liver, and skeletal muscle9-11. The plasma FFAs levels are elevated in most obese people due to an increased release of FFAs from the enlarged and stressed adipose tissues and decreased FFAs clearance12-14. FFAs promote macrophage infiltration and stimulate infiltrated macrophages to release high levels of pro-inflammatory cytokines, such as monocyte chemotactic

Corresponding Author: Qingbo Guan, MD; e-mail: 18004968120@163.com
protein 1 (MCP-1), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)15-17. Meanwhile, these cytokines enhance macrophage infiltration and pro-inflammatory cytokines secretion, and induce adipocyte insulin resistance in a paracrine fashion18,19. Endothelial progenitor cells (EPC) and endothelial cells can also be affected in obesity20-22. The role of endothelial cells is mainly associated with vasculogenesis, the formation of blood vessel wall and the maintenance of blood vessel integrity and permeability. The circulating endothelial cells (CECs) are biomarkers of potential injuries or vascular dysfunction. CECs can be created by various factors including mechanical stress, lack of endothelial cells-membrane interaction, adhesion dysfunction and inflammation. This represents the basic pathological process of the diseases, such as cardiovascular diseases. Besides, FFAs can also cause the apoptosis of endothelial cells, human umbilical vein endothelial cells (HUVECs), human retinal endothelial cells (HRECs), human aortic endothelial cells (HAECs) and EPCs23. Toll-like receptors (TLRs) are a group of proteins that play crucial roles in the innate immune system21. TLRs are usually expressed on immune cells such as dendritic cells (DCs) and macrophages, which are responsible for recognizing various kinds of pathogens and activating immunological responses. The TLRs can be divided into 13 subtypes, from TLR1 to TLR13. Among these subtypes, TLR4 is well known for its recognition of lipopolysaccharide (LPS), which is a symbol of gram-negative bacteria. TLR4 is expressed in most of the cells in human. TLR4 receptor complex is composed of CD-14, an accessory protein and MD-2. In the presence of LPS, the TLR4 receptor complex recruits myeloid differentiation factor-88 (MyD88). The MyD88 activates NF-κB and IKK-β, which in turn activate interleukin-1 receptor-associated kinase (IRAK) that ultimately induces the expression of various inflammatory cytokines. Studies24 have shown that FFAs can also interact with TLR4, leading to its activation or inhibition, which is crucial in some rheumatic diseases, such as rheumatoid arthritis (RA). We investigated the effects of FFAs/TLR4 interaction in HUVECs. FFAs can induce endothelial cell apoptosis; however, no study has been performed on the link between FFAs and inflammation. We suggested that TLR4 could increase the inflammation responses in the presence of FFAs, which might be responsible for the vascular damage caused by obesity.

Materials and Methods

Reagents and Cell Lines

Dulbecco’s Modified Eagle Media (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). Penicillin-streptomycin solution was purchased from HyClone (Shanghai, China). HUVECs and HEK293 cell lines were obtained from Shanghai Type Culture Collection (Shanghai, China). FFAs replaced by palmitic acid for FFAs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against MyD88, NF-κB, IKK-β and β-actin were purchased from Santa Cruz (Santa Cruz, CA, USA). Cells were all cultured with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 5% CO2 and 95% humidity if not specified in the text.

Adenovirus Vector Generation and Infection

HUVECs were infected with constructed adenovirus (Gene Chem Life Technologies, Shanghai, China) to inhibit TLR4 expression. Adenovirus vector was transfected into HUVECs using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Virus expressing only green fluorescent protein (GFP) was used as the blank control group. Infected cells were selected in medium containing 1 mg/mL puromycin after three rounds of infection (6 hours each) in the presence of polybrene (Sigma-Aldrich, St. Louis, MO, USA).

CCK-8 Cell Proliferation and Viability Assay

HUVECs were seeded (2 × 10^3 per well) into 96-well plates and cultured overnight. Culture medium was removed the next day and replaced with fresh medium containing different dosages of FFAs. Cell proliferation and viability were evaluated on day 1 and day 2 using cell counting kit-8 (CCK8, Dojindo Laboratories, Shanghai, China). The cells were treated with CCK-8 solution for 4 h, washed twice with phosphate-buffered saline (PBS) (PH7.2), and incubated at 37°C for 1 to 4 h. OD values were measured at 450 nm using a 96-well plate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry Cell Apoptosis Assay

FFAs induced HUVEC apoptosis was detected by flow cytometry cell apoptosis assay. The sam-
FFAs induced inflammation through TLR4

Samples were quantified on a Becton Dickinson flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using 488 nm excitation and a 600 nm band pass filter. Ten thousand cells in each sample were analyzed. The percentage of apoptotic cells accumulating in the sub-G1 peak was calculated by Cell Quest software.

**Enzyme-linked Immunosorbent Assay (ELISA)**

Levels of murine IL-6, IL-8, CCL5 and CXCL10 in the culture supernatants were measured by ELISA Kit (Abcam, Shanghai, China). The culture supernatants were collected and added to the 96-well plates that were pre-coated with IL-6, IL-8, CCL5 or CXCL10 polyclonal antibody. The samples were immunosorbed by biotinylated polyclonal anti-human IL-6, IL-8, CCL5 or CXCL10 antibody at room temperature for 2 hours. The color development was catalyzed by horseradish peroxidase (HRP) and terminated with 2.5 mol/L sulfuric acid. The absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

**qPCR**

Total RNA was isolated using TRIzol reagent (Life Technologies, South Logan, UT, USA). Reverse transcriptase and oligo(dT) primer were used to prepare cDNA from 1 μg of RNA according to the manufacturer’s instructions (TaKaRa, Dalian, Liaoning, China). 2 μl of each cDNA were used for PCR amplification using primers for MyD88, NF-κB, IKK-β, IL-6, IL-8, CCL5 and CXCL10. The mean cycle threshold (Ct) value for each individual assay was calculated from triplicate measurements and mean Ct values calculated for MyD88, NF-κB, IKK-β, IL-6, IL-8, CCL5 and CXCL10 were normalized by subtraction from the Ct values obtained for the housekeeping reference β-actin (Table I).

**Western Blot**

The proteins were extracted from the cultured cells using radioimmunoprecipitation assay (RIPA) lysis buffer (1% NP40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000 × g for 20 min at 4°C. Protein concentration was determined by using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The lysates were mixed with 4 × SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/L DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min, and then separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane blots were incubated with primary antibodies, including anti-MyD88 (1:1000), anti-NF-κB (1:1000), anti-IKK-β (1:1000) or anti-β-actin (1:1000). β-actin was used as a protein loading control. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA, USA). The autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein bands. The signals were recorded using X-ray film. Images shown in the figures were the representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to loading controls, and then normalized to experimental controls.

**Table I.** Primer sequences for qPCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>Tm (°C)</th>
</tr>
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<tr>
<td>IL-6</td>
<td>5'-GCTTACCCAGGCAAACAT-3'</td>
<td>5'-CTGGCACCAGAAACGA-3'</td>
<td>59</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-GCCGAGGCAGGAC-3'</td>
<td>5'-GGCATCTCCCTGAACG-3'</td>
<td>61</td>
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<tr>
<td>CCL5</td>
<td>5'-AGATGGAGATTTCTGATGGTTGTCCTC-3'</td>
<td>5'-CTTGCTTAGTTCTGTTGCTGG-3'</td>
<td>60</td>
</tr>
<tr>
<td>CXCL10</td>
<td>5'-TACCCACCTAGACAAACGCCCACC-3'</td>
<td>5'-ATCCCAATCAGAAAACCCAGC-3'</td>
<td>60</td>
</tr>
<tr>
<td>MyD88</td>
<td>5'-AGATGACCATGCGGAGGAG-3'</td>
<td>5'-ATCAATCACTCAGGACATTT-3'</td>
<td>58</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5'-CTGTCAGGATG-3'</td>
<td>5'-ACCAGAGAGAAGAAGCAG-3'</td>
<td>60</td>
</tr>
<tr>
<td>IKK-β</td>
<td>5'-AGCAGAAAGAAGAAGCAGGAGGAG-3'</td>
<td>5'-CATCAGGACACAAAGG-3'</td>
<td>61</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-TACCTGTGATGCTCTTG-3'</td>
<td>5'-ATGTCACGCGGAGATT-3'</td>
<td>61</td>
</tr>
</tbody>
</table>

MyD88: Myeloid differentiation factor-88; NF-κB: NF-kappaB; IKK-β: IKK-beta.
Statistical Analysis

All data were analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA) software and the results were showed as mean ± SD. A one-way ANOVA or Student’s t-test was used to assess statistical significance, with \( p \leq 0.05 \) being regarded as significant.

Results

FFAs Treatment Decreased Cell Viability and Increased Cell Apoptosis Rate

HUVECs were treated with different dosages of FFAs for 24 h or 48 h, respectively. Cell viability was evaluated by CCK-8 at the two time points. The results showed that the cell viability of HUVECs treated with 300 μM FAAs was decreased at 24 h (\( p < 0.05 \)). At 48 h, HUVECs treated with all different dosages of FAAs (100 μM, 200 μM and 300 μM) showed significant decrease in cell viability (\( p < 0.05 \)) (Figure 1A, 1B). FCM was performed to test the effects of FFAs on the apoptosis of HUVECs (Figure 1C, 1D). The results showed that both the early (24 h) cell apoptosis rate and the late (48 h) cell apoptosis rate were increased by FFAs treatment (\( p < 0.05 \)) (Figure 1E, 1F).

TLR4 Interference Reversed the Negative Effects of FFAs on HUVECs

To investigate the role of TLR4 in the inflammatory response of HUVECs, adenovirus carrying the inhibitory sequence to TLR4 was constructed. Cells were first infected with the constructed adenovirus and then divided into three groups: 100 μM FAAs treatment group, 200 μM FAAs treatment group and 300 μM FAAs treatment group. The infected cells were then cultured for 24 h or 48 h for cell viability test. The CCK-8 results showed that HUVECs infected with TLR4i adenovirus did not have significant alterations in the cell viability at both 24 h and 48 h compared to the control group (Figure 2A, 2B). We also performed FCM to test the cell apoptosis rate of HUVECs infected with TLR4i adenovirus (Figure 2C, 2D). Consistent with the results of cell viability test, TLR4i adenovirus decreased both early and late cell apoptosis rate induced by FFAs (100 μM) treatments (Figure 2E, 2F).

FFAs Treatment Enhanced Pro-Inflammatory Cytokines Expression

To further explore the change in HUVECs inflammatory responses, ELISA was performed to test the release levels of IL-6, IL-8, CCL5 and CXCL10. The results showed that FFAs treatments (100 μM, 200 μM and 300 μM) significantly increased the release of CCL5 in HUVECs (\( p < 0.05 \)) (Figure 3A). The release level of CXCL10 altered significantly only after 300 μM of FFAs treatment (\( p < 0.05 \)) (Figure 3B). The release levels of IL-6 and IL-8 were significantly increased by FFAs treatments at all concentrations (100 μM, 200 μM and 300 μM) (\( p < 0.05 \)) (Figure 3C, 3D). We also performed qPCR to test the mRNA expression levels of these genes. The relative mRNA expression levels of IL-6, IL-8, CCL5 and CXCL10 were all increased by FFAs treatments (Figure 3E-3H); however, FFAs treatments at 100 μM did not significantly change the mRNA expression levels of CXCL10 and IL-8 (Figure 3F, 3H).

TLR4 Interference Inhibited Pro-Inflammatory Cytokine Expressions Induced by FFAs

To investigate the role of TLR4 in the release levels and mRNA expression levels of IL-6, IL-8, CCL5 and CXCL10, HUVECs were infected with TLR4i adenovirus. The infected cells were divided into three groups: 100 μM FAAs treatment group, 200 μM FAAs treatment group and 300 μM FAAs treatment group. ELISA was performed to test the release levels of IL-6, IL-8, CCL5 and CXCL10. The results showed that TLR4i adenovirus infection significantly decreased the levels of IL-6, IL-8, CCL5 and CXCL10 with different dosages of FFAs treatments (\( p < 0.05 \)) (Figure 4A-4D). qPCR results indicated that the relative mRNA expression levels of these four genes were all down-regulated by the TLR4i adenovirus infection (Figure 4E-4H), which suggested that TLR4 receptor complex played a crucial role in the inflammatory response of HUVECs.

TLR4 Interference Inhibited MyD88, NF-κB and IKK-β Over-Expression Induced by FFAs

To investigate the underlying molecular mechanisms, we measured the level of TLR4 downstream signaling molecules. The WB results showed that MyD88 expression was increased by FFAs treatment at concentrations of 100 μM and 200 μM. The increase in MyD88 was reversed.
FFAs induced inflammation through TLR4

by TLR4i adenovirus infection. The changes in NF-κB and IKK-β protein levels were similar to MyD88 (Figure 5A). Quantitative analysis showed that MyD88, NF-κB and IKK-β expressions were significantly increased compared to the control group, while the adenovirus infection significantly decreased the level of these proteins ($p < 0.05$) (Figure 5B-5D). The mRNA expressions of MyD88, NF-κB and IKK-β were significantly increased (Figure 5E-5G), which were consistent with the WB results, indicating the interaction of FFAs and TLR4 in the inflammation responses.

**Discussion**

We demonstrated that FFAs treatments induced HUVECs apoptosis and increased TLR4-mediat-
Figure 2. TLR4 interference reversed the negative effects of FFAs on HUVECs. (A) Cell viability of HUVECs infected with TLR4i adenovirus after FFAs treatments (100 μM, 200 μM and 300 μM) for 24 h. (B) Cell viability of HUVECs infected with TLR4i adenovirus after FFAs treatments (100 μM, 200 μM and 300 μM) for 48 h. (C) FCM analysis of cell apoptosis of HUVECs infected with TLR4i adenovirus at 24 h post FFAs treatment (100 μM, 200 μM and 300 μM). (D) FCM analysis of cell apoptosis of HUVECs infected with TLR4i adenovirus at 48 h post FFAs treatment. (E) Quantitative analysis of early cell apoptosis rate of HUVECs infected with TLR4i adenovirus. (F) Quantitative analysis of late cell apoptosis rate of HUVECs infected with TLR4i adenovirus. Data in the figures represent mean ± SD (n = 3). *p < 0.05.

Figure 3. FFAs treatment enhanced pro-inflammatory cytokine expressions. (A) ELISA assay of CCL5 release level in HUVECs treated with FFAs. (B) ELISA assay of CXCL10 release level in HUVECs treated with FFAs. (C) ELISA assay of IL-6 release level in HUVECs treated with FFAs. (D) ELISA assay of IL-8 release level in HUVECs treated with FFAs. (E) Relative mRNA expression level of CCL5. (F) Relative mRNA expression level of CXCL10. (G) Relative mRNA expression level of IL-6. (H) Relative mRNA expression level of IL-8. Data in the figures represent mean ± SD (n = 3). *p < 0.05.
ed inflammatory responses, and TLR4 interference significantly reversed these effects. Obesity has become a worldwide health concern because of the negative consequences caused by the early onset of obesity in young children\textsuperscript{25}. The increased body mass index (BMI) is closely related to the increased risk of cardiovascular diseases and diabetes. The prevalence of coronary heart disease (CHD) and ischemic stroke is also increased in people with obesity\textsuperscript{25-27}. Obesity can also increase the overall mortality rate; therefore, detailed pathogenesis of obesity-induced diseases is worth studying. FFAs, also called uncombined fatty acids or FFAs, are the products of triglyceride breakdown. FFAs are insoluble in water and they are bound to plasma protein albumin for transportation. The FFAs levels in plasma are elevated in most obese people because of the increased release of FFAs from the enlarged and stressed adipose tissues and decreased FFAs clearance\textsuperscript{28-30}. Once the FFAs levels are elevated in the circulation system, FFAs will inhibit insulin's antilipolytic action and the level of FFAs will be further increased\textsuperscript{30}. In this work, we focused on the effects of FFAs treatment on the inflammatory responses in HUVECs. Previous researches have reported that non-esterified fatty acids could modify the inflammatory response and eicosanoid biosynthesis in bovine endothelial cells\textsuperscript{31}. The increase of FFAs level is related with the insulin resistance and inflammation in HUVECs. The results showed that FFAs could induce endothelial cell apoptosis. The functional relationship between inflammation and apoptosis is complex and is related to different stimuli and cell line types. Pro-inflammatory cytokines play important roles in cellular apoptosis in inflammatory diseases. A study\textsuperscript{32} showed that ectopically expressed NLR family CARD domain-containing protein 4 (NLRC4) and apoptosis speck-like protein (ASC) in HEK293T cells induce apoptosis, in which NLRC4 and ASC form a complex to recruit endogenous caspase-8 and induce apoptosis. Caspase-8 is associated with inflammasome responses. In addition to its role in mediating inflammasome-associated apoptosis, caspase-8 also mediates transcriptional upregulation of pro-IL1β in response to TLR4 engagement, hence serving as a checkpoint for efficient inflammasome-induced cytokine responses\textsuperscript{33}. The interaction of pro-inflammatory cytokine activation and cell death was further elucidated by research showing that the extrinsic apoptotic caspase, caspase-8 or caspase-1, directly processed pro-inflammatory cytokines, activated the NLRP3 inflammasome, or bound to inflammasome complexes to induce apoptotic cell death\textsuperscript{34}. Meanwhile, apoptosis promotes inflammatory cytokine expressions by the interaction of multiple genes and signaling pathways. Apoptosis-inducing signaling comp-

**Figure 4.** TLR4 interference inhibited pro-inflammatory cytokine expressions induced by FFAs. HUVECs were first infected with TLR4i adenovirus to interfere TLR4 expression. (A) ELISA assay of CCL5 release level in HUVECs treated with FFAs. (B) ELISA assay of CXCL10 release level in HUVECs treated with FFAs. (C) ELISA assay of IL-6 release level in HUVECs treated with FFAs. (D) ELISA assay of IL-8 release level in HUVECs treated with FFAs. (E) Relative mRNA expression level of CCL5. (F) Relative mRNA expression level of CXCL10. (G) Relative mRNA expression level of IL-6. (H) Relative mRNA expression level of IL-8. Data in the figures represent mean ± SD (n = 3). *p < 0.05.
plexes activate the apoptotic initiators caspases-8 and caspases-1035. Caspase-9-activating apoptosis promotes oligomerization of inactive procaspase-1 zymogens in inflammasomes, which results in the proximity-induced autoactivation of the protease36. Caspase-1 maturation and release of the pleiotropic inflammatory cytokines interleukin (IL)-1β and IL-18, thereby playing important roles in inflammatory and immune responses37. Furthermore, inflammasome-induced cell death is increasingly recognized to the extend well beyond caspase-1-induced pyroptosis, and encompasses apoptosis and pyronecrosis. Circulating endothelial progenitor cells (EPCs), stem cell-derived endothelial cells, exhibit potentials in the treatment of cardiovascular diseases38,39. Evidence showed that EPCs could increase the proliferation and enhance the endothelium regeneration in vein grafts, while the vascular endothelial cells could repair the damaged en-

Figure 5. TLR4 interference inhibited MyD88, NF-κB and IKK-β over-expressions induced by FFAs. (A) Representative WB images of MyD88, NF-κB, IKK-β and actin. (B) Quantitative analysis of MyD88 expression against actin. (C) Quantitative analysis of NF-κB expression against actin. (D) Quantitative analysis of IKK-β expression against actin. (E) Relative mRNA expression level of MyD88. (F) Relative mRNA expression level of NF-κB. (G) Relative mRNA expression level of IKK-β. Data in the figures represent mean ± SD (n = 3). *p < 0.05 compare to control group, ’p < 0.05 compare to FFAs treated groups.
FFAs induced inflammation through TLR4

We demonstrated that FFAs increased apoptosis rate and inflammatory responses in HUVECs. Silencing TLR4 significantly reversed the harmful effects of FFAs indicating that TLR4 might be a potential target in the treatment of FFAs induced inflammation and damage.

Conclusions

We demonstrated that FFAs increased apoptosis rate and inflammatory responses in HUVECs. Silencing TLR4 significantly reversed the harmful effects of FFAs indicating that TLR4 might be a potential target in the treatment of FFAs induced inflammation and damage.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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