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Phosphorylation of tyrosine-14 on Caveolin-1 enhances lipopolysaccharide-induced inflammation in human intestinal Caco-2 cells

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Abstract Caveolin-1 (Cav-1) is the main structural component of the caveolae on the plasma membrane, which regulates various cellular processes, including cell growth, differentiation, and endocytosis. Although a recent study demonstrated that Cav-1 might be involved in diabetes-associated inflammation, its exact role in the intestine was unclear. In this study, we examined the intestinal expression of Cav-1 in diabetic conditions. We also investigated its effect on lipopolysaccharide (LPS)-induced inflammation by expressing this protein in human intestinal Caco-2 cells lacking Cav-1. We observed that increased Cav-1 levels and decreased expression of tight junction proteins affected intestinal permeability in high-fat diet-induced diabetic mice. When Caco-2 cells were treated with LPS, Cav-1 enhanced the NF-κB signaling. Moreover, LPS reduced the expression of tight junction proteins while it increased cell-cell permeability and reactive oxygen species generation in Caco-2 cells and this effect was amplified by cav-1 overexpression. LPS treatment promoted phosphorylation of tyrosine-14 (Y14) on Cav-1, and the LPS-induced NF-κB signaling was suppressed in cells expressing non-phosphorylatable Cav-1 (tyrosine-14 to phenylalanine mutant), which reduced intestinal barrier permeability. These results suggest that Cav-1 expression promotes LPS-induced inflammation in Caco-2 cells, and phosphorylation of Y14 on Cav-1 might contribute to the anti-inflammatory response in LPS-induced NF-κB signaling and cell permeability.

Keywords Caveolin-1 · High fat diet · Intestine · Permeability · Tight junction protein

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Introduction

Alteration of gut microbiota and increased lipopolysaccharide (LPS) levels due to a high-fat diet induce intestinal permeability, causing endotoxemia [1,2]. Endotoxemia-induced inflammation increased cytokine levels in the liver, muscle, pancreas, and adipose tissue, resulting in chronic metabolic diseases, including diabetes and obesity [3-5].

The intestinal epithelial barrier is composed of apical multiple protein complexes and maintained by tight junction (TJ) proteins, including zonula occludens-1 (ZO-1), occludins, and claudins [6,7]. The TJ proteins transport water/ions through the epithelial paracellular pathway and prevent the invasion of immunogenic macromolecules [8,9]. Therefore, the loss of these proteins due to various factors increases intestinal permeability, contributing to the progression of systemic inflammation.

Caveolins (Cav) are the main components of caveolae, which are 50-100 nm plasma membrane invaginations. There are three Cav isoforms (Cav-1, -3), of which Cav-1 and -2 are generally co-expressed (adipocyte, endothelial cell, and fibroblast), whereas Cav-3 is muscle-specific [10]. Cav-1 is crucial for caveolae formation, and it binds to molecules involved in various signaling pathways, including cell migration, cell proliferation, cell membrane oligomerization, and vesicular transport [11]. Cav-1 can bind to toll-like receptors (TLR), such as TLR4 and TLR5, and its interaction with TLR4 in the endothelium activates nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and the

production of proinflammatory cytokines [12].

Cav-1 is phosphorylated on tyrosine 14 (Y14) by the Src family tyrosine kinases, Src, Abl, or Fyn [13,14]. Studies observed that this phosphorylation occurs in response to proinflammatory mediators and is considered a marker for cell death or inflammation promoter [15]. Previously, we reported that increased levels of Cav-1 and phosphorylation of Y14 on cav-1 in the cytokine mixture-treated cells. Moreover, siRNA-mediated downregulation of Cav-1 inhibited the cytokine-induced NF-κB activation and apoptosis of pancreatic beta-cells along with increased glucose-stimulated insulin secretion [16].

Although Cav-1 is normally present in the intestine and regulates lipid or cholesterol metabolism, the association between Cav-1 expression and enhanced sensitivity to inflammation in intestinal cells is unexplored. Therefore, we investigated the changes in Cav-1 expression in the intestines of high-fat diet (HFD)-fed mice and tested whether the presence of Cav-1 modulates LPS-induced inflammation in a human intestinal cell model, Caco-2 cells.

Materials and Methods

Animal and intestine collection

Four-week-old male C57BL/6J mice were purchased from Korea Research Institute of Bioscience & Biotechnology (KRIBB, Daejeon, South Korea) and Daehan Biolink (Daehan Biolink Co. LTD., Eumsung, South Korea). After environmental adaptation for 7 d, the high-fat diet (HFD, 60% fat, D12492; Research Diets, New Brunswick, NJ, USA, n =8) and the normal fat diet (NFD, 4.5% fat, Purina, n =6) were supplied to the mice for 12 weeks. At the end of the experimental period, the intestines were obtained for morphological analysis or protein expression analysis. All experiments were approved by the Institutional Animal Care and Use Committee of Eulji University (EUIACUC18-7) and confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

Cell culture

Caco-2, human intestinal cells were grown in Minium essential medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Madison, WI, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Welgene Inc., Daegu, South Korea), 25 mM HEPES (Thermo Fisher Scientific) at 37 °C in a humidified chamber containing 95% air and 5% CO_2.

Transfection

Caco-2 cells were transfected with 5 μg empty vector (pcDNA3.1), pcDNA3.1-cav-1 (Thermo Fisher Scientific) and pcDNA3.1-cav-1-Y14F (non-phosphorylated; phenylalanine substitution of tyrosine). Lipofectamine 2000 reagent (Invitrogen, CA, USA) was used for transfection according to the manufacturer's instructions. After 24 h, the medium was replaced with 1 μg/mL LPS for various time periods.

Western blotting

Mouse intestinal tissue was lysed in mammalian protein extraction buffer containing protease inhibitor cocktails. Caco-2 cells were extracted from cytoplasm and nuclear proteins using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific), including protease inhibitor cocktails. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane. Antibodies used for western blotting were anti-caveolin-1 (1:1000; #610406; BD biosciences, Cambridge, UK), anti-phospho-cveolin-1 (pY14) (1:1000; #611338; BD biosciences), anti-NFκB (1:1000; #8242; cell signaling technology, MA, USA), anti-p-NFκB (1:1000; #3033; cell signaling technology), anti-IκB (1:1000; #4814; cell signaling technology), anti-p-IκB (1:1000; #2859; cell signaling technology), anti-ZO-1 (1:1000; #40-2200; Invitrogen), anti-claudin-1 (1:1000; #37-4900; Invitrogen), anti-occludin (1:1000; #40-4700; Invitrogen), p-SRC (1:1000; #2101; cell signaling), anti-NRF2 (1:1000; #33649; cell signaling technology), anti-actin (1:2500; #47778; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-Lamin B1 (1:1000; #374015; Santa Cruz Biotechnology). Signals were detected using a Fujifilm luminescent image analyzer (LAS 4000) with an enhanced chemiluminescence detection kit (Millipore; Billerica, MA, USA). The bands were quantified using Image J software.

Electrical resistance measurements

The trans-epithelial electrical resistance (TEER) was measured using an ohmmeter with chopstick electrodes (EVOM2, World Precision Instruments, Sarasota, FL, USA). Caco-2 cells were seeded on a transmembrane, the apical side of a 12 well transwell plate, at 2.5×10^4 cells/well, and 1.5 mL of medium was added to the basolateral side. The medium was changed once every 2-3 days for 2 weeks. When the cells were proliferated to monolayers, epithelial electrical resistance was performed after pcDNA3.1, pcDNA3.1-cav-1, and pcDNA3.1-cav-1-Y14F transfection for 24 h. After washing three times with 0.5 mL of Dulbecco's phosphate buffered saline (D-PBS), Hank's balanced salt solution (HBSS) was changed to 0.5 mL in the apical side and 1.5 mL HBSS in the basolateral side, and then incubated in a CO_2 incubator for 30 min. The electrical resistance value between the apical side and basolateral side was measured. The upper layer of HBSS was suctioned and then treated with HBSS at a concentration of 1 μg/mL LPS, and the TEER was measured every 2 h.

Immunohistochemistry

Small intestine samples were fixed in 10% buffered formalin for 48 hours, embedded in paraffin, and sliced into 4-μm-thick sections. Sections were deparaffinized with xylene and stained with hematoxylin. We then performed immunohistochemistry using primary antibody to Cav-1 (1:100; #610406; BD biosciences),

ZO-1 (1:100; #40-2200; Invitrogen), occludin (1:100; #40-4700; Invitrogen), and claudin-1 (1:100; #37-4900; Invitrogen). For qualitative identification of antigens by light microscopy, we used the liquid DAB+ substrate chromogen system (Dako, Carpinteria, CA, USA). Sections were observed under microscope, cav-1 and junction proteins expression were also quantified using ImageJ software. Briefly, immunohistochemistry images were acquired using identical parameters and background was subtracted, and the staining intensity was measured.

Reactive oxygen species (ROS) measurement

Fluorogenic dye 2,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA, Invitrogen) was used for ROS measurement. Caco-2 cell were plated at 1.0×10^6 cells/well in 6 well plate and incubated for 24 h. Cav-1 transfection was performed for 24 h. After LPS was treated for 1 h, and then washed with D-PBS. The cells were treated with 5 μM H2-DCFDA in 37 ºC CO_2 incubator for 15 min and harvested using trypsin-EDTA. After centrifugation, the supernatant was discarded, cells were resuspended in cold fluorescence- activated cell sorting (FACS) buffer (1% FBS in D-PBS) containing 8% NBF, fixed in the dark for 20-30 min. After centrifugation again, the supernatant was discarded, cells were resuspended with 0.35 mL of FACS buffer. Then, intracellular fluorescent compounds (2′,7′-dichlorofluorescein, DCF) were measured by FACS analysis (FACS Calibur; BD Biosciences).

Statistical analysis

Results are expressed as mean ± SD of three separate experiments. Data were analyzed using analysis of variance followed by post-hoc analyses using the Tukey range test (SPSS 10.0 statistical software, SPSS, Chicago, IL, USA). $p<0.05$ indicated a statistically significant difference between groups.

Results

Expression of TJ proteins and Cav-1 in the intestinal epithelium of HFD mice

As HFD decreases the expression of TJ proteins [17], we used Western blotting to evaluate the intestinal levels of claudin-1 and ZO-1 in 12-week-old HFD-fed mice. As shown in Fig. 1A and B, the level of these proteins was significantly lower in HFD-fed mice than in mice fed with a normal-fat diet (NFD). The histological results using immunostaining also showed reduced

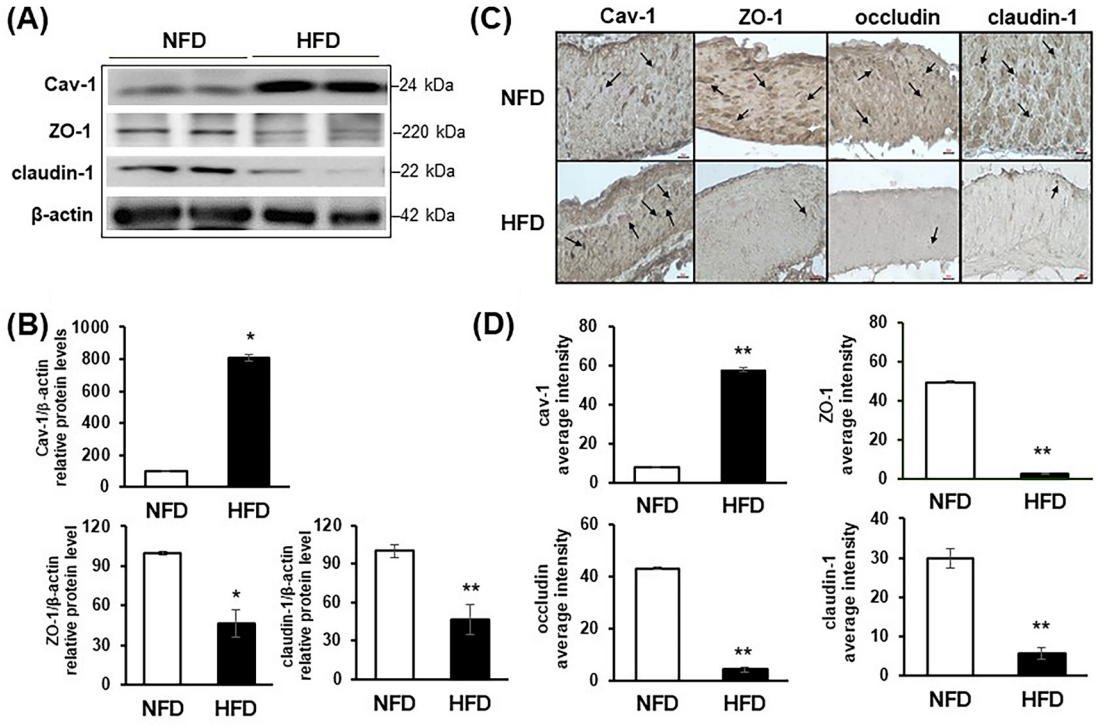

Fig. 1 Expression level of tight junction protein and cav-1 in intestinal epithelium from high fat diet mice. Mice were fed with NFD (n =6) and HFD (n =8) for 12 weeks. (A) Representative immunoblot of cav-1, ZO-1 and claudin-1. (B) The densities of western blot signals were measured and the relative expression level was normalized to that of b-actin. (C) Immunohistochemistry staining (magnification, 200×) for cav-1, ZO-1, occludin and claudin-1 in intestine of NFD and HFD-induced mice (bar =10 μm). The black arrow in the stained section indicates the dark brown dots. (D) Quantification of cav-1 immunohistochemistry were performed using image J to determine the staining site and total area, and calculated as staining site/total area * 100%. Values are means ± SD from triplicate experiments, *$p<0.05$, **$p<0.01$ vs. NFD. NFD, normal-fat diet; HFD, high-fat diet; cav-1, caveolin-1.; ZO-1, zonula occludens-1

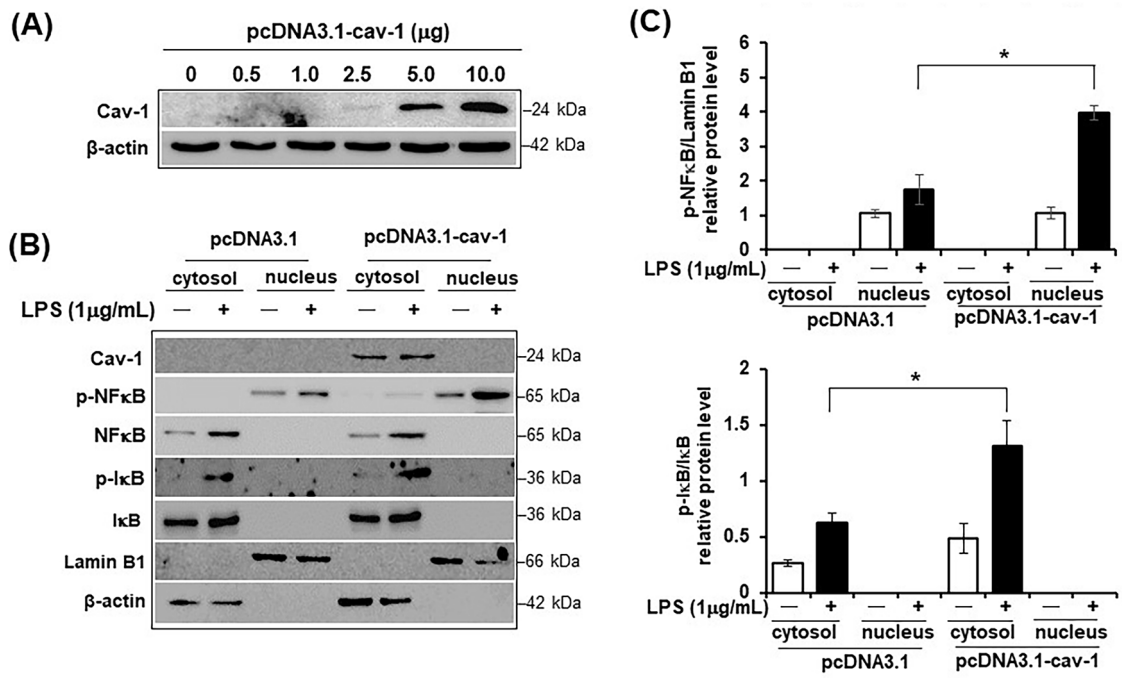

Fig. 2 Overexpression of Cav-1 enhanced NF-κB activation in LPS-treated Caco-2 cells. (A) After transfection of pcDNA3.1-cav-1 to various concentrations (0.5, 1, 2.5, 5 and 10 μg) in Caco-2 cells, cell lysates were subjected to western blot with specific antibodies. β-Actin was used as the loading control. (B) LPS (1 μg/mL) was treated with pcDNA3.1- or pcDNA3.1-cav-1-transfected Caco-2 cells for 30 min. After cytosol/nuclear fraction, protein expression level of NFκB signals and cav-1 was determined by Western blot. (C) The densities of western blot signals were measured, and the relative expression level was normalized to that of lamin B1 and the nonphosphorylated form (IκB). Values are means ± SD from triplicate experiments, *p <0.05

expression of these proteins, and their quantification indicated significantly lower levels in the HFD-fed mice than in the NFD-fed mice (Fig. 1C, D). The intestinal Cav-1 levels in the HFD-fed mice were significantly higher than in the NFD-fed mice (Fig. 1A, B). The dark brown dots indicate higher Cav-1 expression in HFD-fed mice, and the amount of Cav-1 was also significantly higher in the intestines of HFD-fed mice than in NFD-fed mice (Fig. 1C, D).

Overexpression of Cav-1 enhanced NF-κB activation in LPS-treated Caco-2 cells

To investigate whether increased Cav-1 expression in the intestine of HFD-induced mice is involved in inflammation, we treated Caco-2 cells, a human epithelial cell line, with LPS to induce NF-κB activity [18]. As the basal Cav-1 levels are very low in Caco-2 cells, we transfected pcDNA3.1-cav-1 into these cells and examined the signaling pathways with or without 1 μg/mL of LPS. The Caco-2 cells were transfected with various concentrations (0.5, 1, 2.5, 5, and 10 μg) of pcDNA3.1-cav-1, and 10 μg of DNA induced cell toxicity. Therefore, we used 5 μg DNA in this experimnets (Fig. 2A). Further, 24 h after transfection with the pcDNA3.1 and pcDNA3.1-cav-1 vectors, the cells were treated with LPS (1 μg/mL) for 30 min, and the nucleus/cytoplasm was fractionated to examine the expression of NF-κB signaling pathway-related proteins. Figure 2B indicates that normal overexpression was induced by the pcDNA3.1-cav-1 vector and LPS increased NF-κB phosphorylation in the nuclear fraction of pcDNA3.1-transfected cells, which was also significantly increased by Cav-1 overexpression (Fig. 2B). The p-NF-κB/laminB ratio was significantly increased (by 2-fold) after LPS treatment of the cells overexpressing Cav-1 (Fig. 2C). Phosphorylated IκB was observed in the cytosolic fraction of the Cav-1 overexpressing cells compared with the control vector-transfected cells (Fig. 2B, C).

Overexpression of Cav-1 increased permeabilization in LPS-treated Caco-2 cells

To determine whether Cav-1 overexpression affects permeabilization, we measured the levels of TJ-related proteins in LPS-treated Caco-2 cells with or without the Cav-1 expression vector. Western blot results showed that ZO-1, occludin-1, and claudin-1 levels were significantly decreased after LPS treatment. Moreover, cells overexpressing Cav-1 showed lower levels of these proteins than cells transfected with pcDNA3.1 vector only after treatment with LPS (Fig. 3A, B). Next, to determine whether Cav-1 impaired the intestinal epithelial barrier function, the TEER was measured in the LPS-treated Caco-2 cells using an ohmmeter with chopstick electrodes. Time zero TEER (0 h) values were measured before Cav-1 upregulation, and time-dependent TEER values at 2, 4, 6,

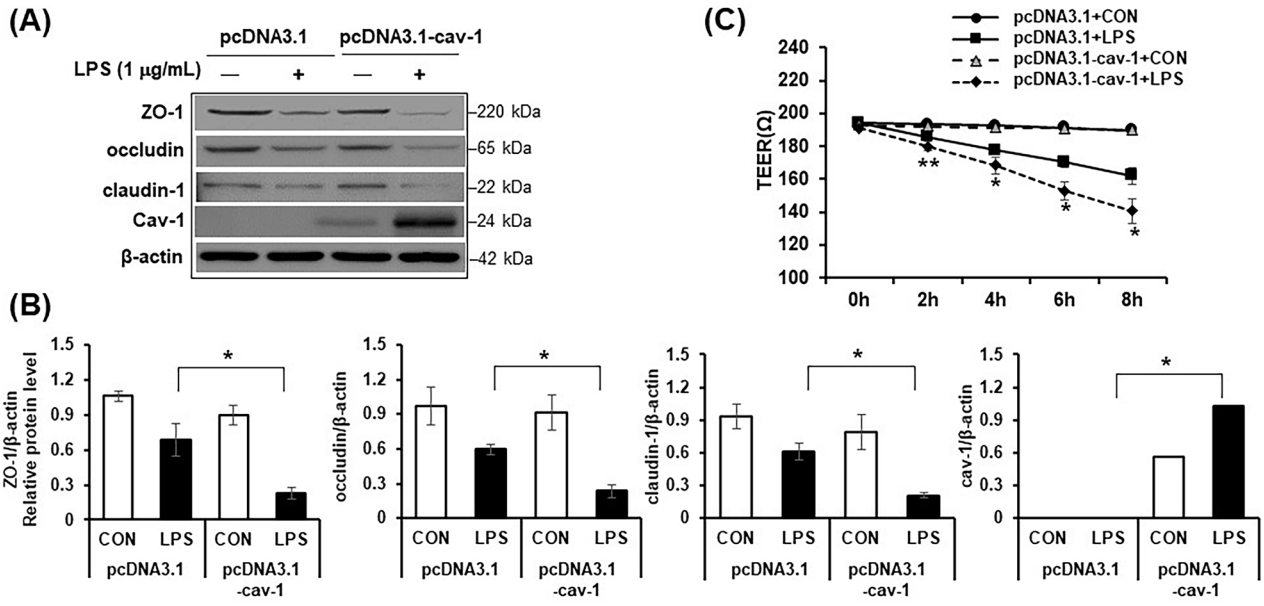

Fig. 3 Overexpression of Cav-1 increased permeabilization in LPS-treated Caco-2 cells. (A) Caco-2 cells were transfected with pcDNA3.1 or pcDNA3.1-cav-1. After LPS (1 μg/mL) treatment for 24 h, proteins were detected by western blot. (B) The densities of western blot signals were measured and the relative expression level was normalized to that of b-actin. (C) TEER (Ω) for cav-1 overexpression Caco-2 monolayers were monitored for up to 8 h at 2 h intervals after LPS (1 μg/mL) treatment. Values are means ± SD from triplicate experiments, *p <0.05, **p <0.01 vs. pcDNA3.1 LPS

and 8 h were measured after exposing cells with or without the Cav-1 expression vector to LPS. As shown in Fig. 3C, the TEER values were significantly decreased in the LPS-treated cells (to 20% of the controls) after 8 h of treatment, indicating increased permeabilization of Caco-2 cells. The cells overexpressing Cav-1 showed attenuated electrical resistance, as indicated by the significantly lower TEER values than cells transfected with the pcDNA3.1 control vector after LPS treatment (Fig. 3C).

Overexpression of Cav-1 increased ROS generation in LPS-treated Caco-2 cells

As ROS generation disrupts the integrity of the Caco-2 cell monolayer [19], we examined the dichlorodihydrofluorescein diacetate (DCF-DA) levels in the Cav-1 overexpressing cells using FACS analysis (Fig. 4A). We observed that while the cells transfected with the pcDNA3.1 control vector showed a negligible increase in ROS generation, the ROS levels were significantly higher (2-fold, p <0.01) in the Cav-1 overexpressing cells than those transfected with the control vector (Fig. 4B). We next determined the protein expression of NRF2, which is widely expressed in various tissues as a transcription factor responsible for the modulation of inflammation response under ROS condition [20]. Under stressed condition, NRF2 translocates to the nucleus and induces the transcriptional activation of target genes with antioxidnat response elements (AREs) in their gene regulatory regions [21]. As shown in the results in Fig. 4C and 4D, LPS increased NRF2 translocation to the nucleus, which was further facilitated by overexpression of cav-1 (p <0.05).

Phosphorylation of Y14 on Cav-1 promotes LPS-induced NF-κB signaling and intestinal barrier dysfunction in Caco-2 cells

Oxidative stress is associated with the phosphorylation of Y14 on Cav-1 by the Src family kinases [13,14], leading to increased transcellular permeability [22]. Therefore, we evaluated Cav-1 phosphorylation by LPS treatment and the involvement of Src family kinases. Figure 5A shows that LPS treatment of Caco-2 cells overexpressing Cav-1 for 30 min increased Y14 phosphorylation (Fig. 5A). Moreover, LPS-induced phosphorylation was inhibited by SU6656, an Src inhibitor (Fig. 5A). Next, to examine whether phosphorylation of Y14 on Cav-1 by the Src family kinases promotes LPS-induced inflammation, Caco-2 cells were transfected with wild-type (pcDNA3.1), Cav-1 overexpression or Cav-1 Y14F mutant (pcDNA3.1-cav-1 Y14F) plasmids and the expression of the IκB signaling were measured. We observed that 24 h post-transfection, Cav-1 phosphorylation was significantly higher in the Cav-1 overexpressing cells than in the control vector-transfected cells after LPS treatment. Conversely, this was inhibited in the cells carried with pcDNA3.1-cav-1 Y14F. Moreover, LPS treatment significantly attenuated the phosphorylation of IκB in the Cav-1 Y14F mutant cells compared with the cells expressing the Cav-1 and control vector (Fig. 5B, C). Finally, evaluation of the TEER value in cells overexpressing cav-1 and its mutant protein after LPS treatment showed that the Cav-1 Y14F mutant expressing cells showed decreased sensitivity to LPS (Fig. 5D).

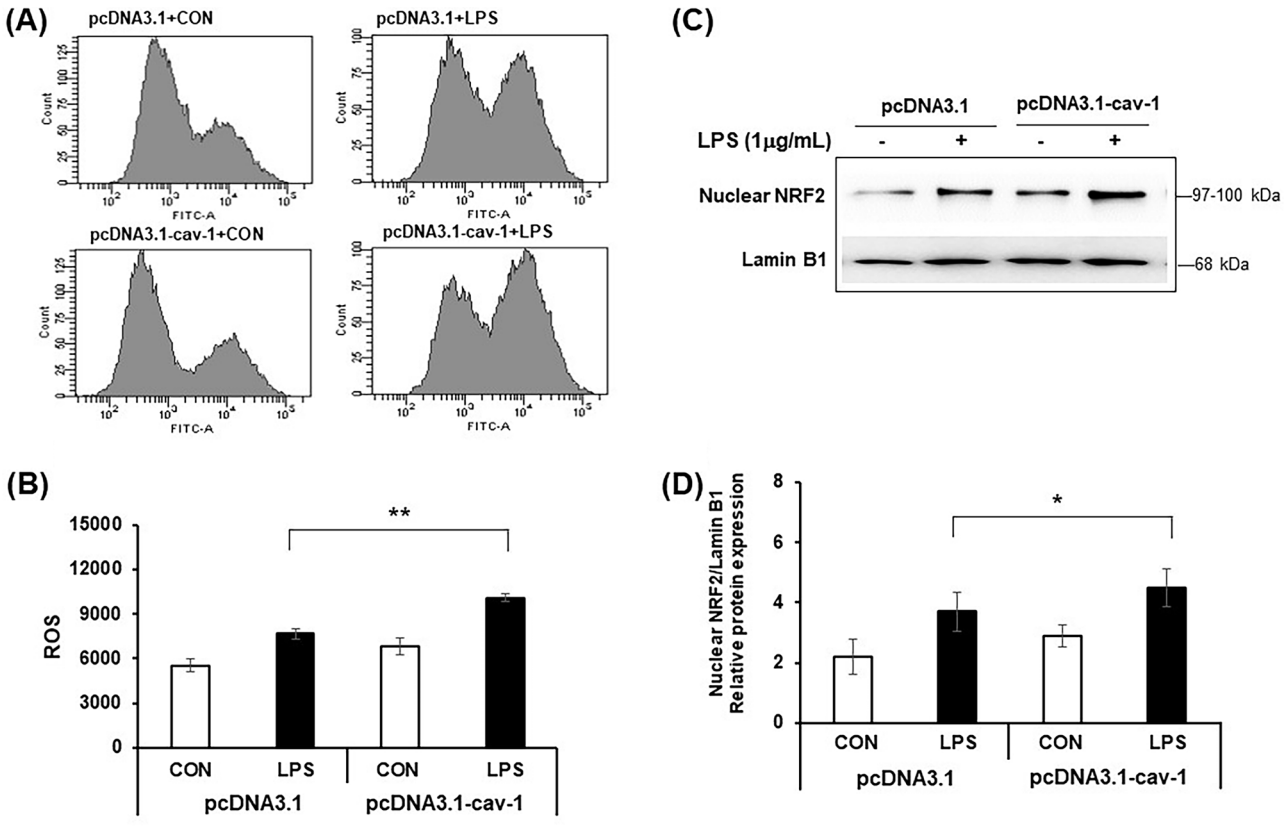

Fig. 4 Overexpression of cav-1 increased ROS generation in LPS-treated Caco-2 cells. (A) The cells were transfected with pcDNA3.1 and pcDNA3.1-cav-1 for 24 h followed by treatment with LPS (1 μg/mL) for 1h. The production of intracellular ROS was measured using DCF-DA by flow cytometry. (B) Quantitative data demonstrating the DCF-DA positive cells. (C) The nuclear NRF2 was detected by western blot. (D) The densities of nuclear NRF2 signals were measured and the relative expression level was normalized to that of Lamin B1. Values are means ± SD from triplicate experiments, *p <0.05, **p <0.01 vs. pcDNA3.1 LPS

Discussion

HFD increases the intestinal absorption of LPS, which triggers postprandial endotoxemia [23], consequently inducing insulin resistance in the liver, muscles, pancreas, and adipose tissues. Therefore, preventing LPS-induced increase in intestinal permeability or suppressing its related signaling pathways might be an important therapeutic target for treating diabetes related inflammation.

Cav-1 mRNA expression was higher in the adipose tissues of obese patients with type-2 diabetes than in normal controls [24]. Moreover, increased caveolar expression was also observed in the lung endothelium of diabetics [25]. Cav-1 overexpression in the pancreatic beta cells enhanced palmitate-induced apoptosis [26] and recruitment of cytokine receptors to the caveolae, resulting in cytokine-induced beta cell apoptosis [16]. Here, we found higher Cav-1 levels in the intestines of HFD-fed mice than NFD-fed mice, suggesting the involvement of intestinal Cav-1 expression in endotoxemia-induced inflammation.

Although Cav-1 is normally expressed in murine intestines [27], our results showed negligible Cav-1 levels in Caco-2 cells, consistent with previous reports [16]. This might be because Cav-1 expression is more involved in cell death under stress than in basic cellular functions.

NF-κB is a nuclear transcription factor that causes intestinal inflammation and is activated by proinflammatory mediators, including tumor necrosis factor-α, interleukin (IL)-1β, interferon-γ and LPS. Studies observed that the expression of TLR-4, an LPS-binding pattern recognition receptor in the intestinal tissues, is significantly increased in irritable bowel disease. The TLR-4-LPS complex phosphorylates IκB-α through an intracellular signaling cascade [28]. We found that LPS-induced activation of NF-κB is more potent when Cav-1 is overexpressed. Previous reports also suggested that LPS-induced IκB degradation and NF-κB activation were significantly reduced in the lungs of Cav-1 knock-out mice [29]. Moreover, silencing Cav-1 expression inhibited cytokine-mediated NF-κB activation and expression of genes involved in inflammation [16], indicating that Cav-1 expression enhanced the inflammation response and LPS sensitivity in Caco-2 cells.

Cav-1 is also involved in regulating the assembly of TJ proteins, which further influences the function of the intestinal epithelial barrier [30]. Guillermo Area et al. reported increased

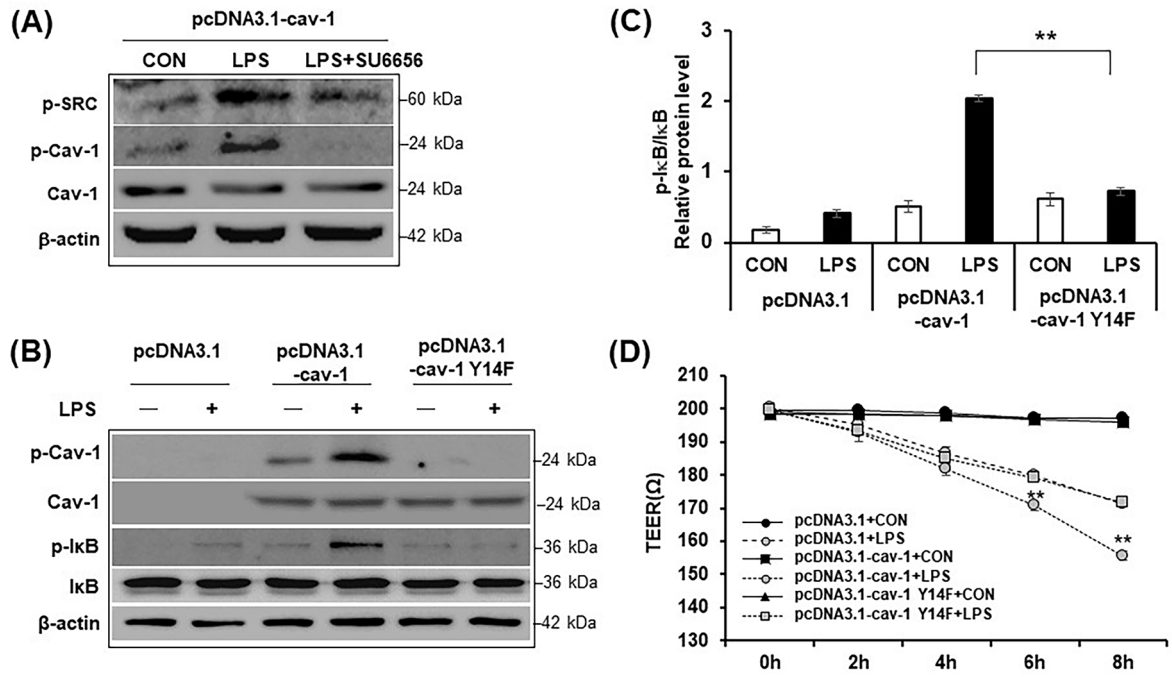

Fig. 5 Cav-1 phosphorylation on tyrosine-14 is required to promote LPS-induced NF-κB signaling and intestinal barrier dysfunction in Caco-2 cells. (A) Caco-2 cells were treated with LPS (1 μg/mL) with or without SU6656 (5 μM) and expression level of p-SRC, p-Cav-1 and cav-1 was measured by Westernblot. (B) Caco-2 cells were transfected with pcDNA or cav-1 or cav-1 Y14F. After LPS (1 μg/mL) treatment for 0.5 h, proteins were detected by western blot. (C) The densities of western blot signals were measured and the relative expression level was normalized to that of nonphosphorylated form (IκB). (D) After transfection of pcDNA, cav-1, and cav-1-Y14F, TEER (Ω) for overexpression caco-2 monolayers were monitored for up to 8 h at 2 h intervals after LPS (1.0 μg/mL) treatment. Values are means ± SD from triplicate experiments, $^{**}p$ <0.01 vs. cav-1 Y14F LPS

binding between Cav-1 and claudin-2 in a necrotizing enterocolitis rat model [31]. Nag et al. also demonstrated that increased Cav-1 expression preceded the decrease in the occludin and claudin-5 levels during blood-brain barrier breakdown [32]. Zhang et al. showed that type 1 immunodeficiency virus (Tat) elevated Cav-1 expression in human brain microvascular endothelial cells, which sequentially downregulated the occludin, ZO-1, and ZO-2 levels [33]. We also found a higher reduction in the TJ protein levels in the Cav-1 overexpressing cells compared with the control cells, resulting in a lower TEER value in these cells after LPS treatment. These results suggest that Cav-1 negatively regulates the TJ-associated proteins. However, these effects of the signaling pathway involved in Cav-1-mediated regulation of the permeability and integrity of intestinal cells need to be studied further.

Intestine cells are sensitive to inflammatory processes and to ROS levels exceeding the total antioxidant capacity [34]. Previous studies revealed that LPS induces excess ROS production, which causes oxidative stress and, subsequently, cell damage [35]. In our study, LPS stimulation significantly increased ROS generation, which was further enhanced by Cav-1 overexpression. When we observed NRF2 translocation by ROS generation, NRF2 translocated from the cytoplasm to the nucleus after overexpression of cav-1 in Caco-2 cells, indicating that NRF2 activation is cav-1 dependent. Consistent with our results, other studies have shown that Cav-1 induces oxidative stress. Based on the free radical theory of aging, Cav-1-mediated oxidative stress exerts deleterious cellular senescence events, such as aging and age-related diseases [36].

In this study, we observed that LPS treatment significantly increased phosphorylation of Y14 on Cav-1, which was abolished by preincubation with the Src kinase inhibitor SU6656. The phosphorylation of Y14 on Cav-1 is vital for the response to various stimuli [37,38]. Cav-1 expression promotes palmitate-induced apoptosis which involves Y14 phosphorylation by Src family kinases [26]. Wang N et al. also reported that LPS-induced Cav-1 phosphorylation increases the transcellular permeability in a Src-dependent manner in the pulmonary microvascular endothelial cells [22]. This suggests that LPS-induced Cav-1 phosphorylation is Src-dependent, consistent with previous reports.

As phosphorylation of Y14 on Cav-1 is known to occur in response to oxidative stress [39], we evaluated its relevance by creating a mutant in which the Y14 is replaced with a non-phosphorylatable amino acid phenylalanine (F). Caco-2 cells transfected with this mutant showed diminished LPS sensitivity, similar to the pcDNA3.1 vector-transfected cells. Moreover, LPS-induced impairment of barrier integrity was ameliorated in the Y14F-transfected cells. This implies that phosphorylation of Y14 on Cav-1 contributes to enhanced LPS sensitivity, especially in Cav-1 overexpressing Caco-2 cells in response to ROS generation.

In conclusion, phosphorylation of Y14 on Cav-1 is integral for increasing intestinal permeability via enhanced NF-κB signaling, increased ROS generation, and reduced TJ proteins induced by LPS in Caco-2 cells. These results suggest that phosphorylated Cav-1 is an important target in LPS-induced intestinal inflammation.

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