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1,25-Dihydroxyvitamin D3 preserves intestinal epithelial barrier function from TNF-α induced injury via suppression of NF-kB p65 mediated MLCK-P-MLC signaling pathway

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ABSTRACT

Substantial studies have demonstrated the protective effect of 1,25-Dihydroxyvitamin D3 (1,25(OH)2D3) on intestinal barrier function, but the mechanisms are not fully illustrated. In this study, the effect of 1,25(OH)2D3 on TNF-α induced barrier dysfunction was further investigated in Caco-2 cell monolayers. The barrier function of Caco-2 monolayers was evaluated by measuring trans-epithelial electrical resistance (TEER) and FITC-Dextran 40,000 Da (FD-40) trans-membrane flux. ZO-1 and Occludin were chosen as markers of the localization of tight junction (TJ) proteins for immunofluorescence. The expression of MLCK and phosphorylation level of myosin light chain (MLC) were measured by immunoblotting. The activation of NF-kB p65 was analyzed by EMSA and immunofluorescence. The results suggest that 1,25(OH)2D3 preserves intestinal epithelial barrier function from TNF-α induced injury via suppression of NF-kB p65 mediated activation of MLCK-P-MLC signaling pathway.

1. Introduction

An intact intestinal epithelial TJ barrier functions as a physical barrier between the luminal content and the internal environment. Injury of TJ has been shown to be an important pathogenic factor of multiple enteropathies by allowing paracellular flux of luminal noxious content that initiates and propagates inflammatory responses [1,2]. Plethora of inflammatory cytokines including TNF-α have been described to induce injuries of the intestinal epithelial barrier function [3,4]. Although the underlying mechanism remains are not fully illustrated, the altered localization of TJ proteins produced by the activation of MLCK-P-MLC signaling pathway is believed to play a vital role in the TNF-α induced injury of intestinal barrier function [5,6].

1,25(OH)2D3 was found to be able to protect the intestinal barrier from injuries induced by multiple reagents [7–10]. However, the mechanisms underlying the protective effect of 1,25(OH)2D3 on intestinal barrier function remain essentially unknown. This study reveals that 1,25(OH)2D3 preserves intestinal epithelial barrier function from TNF-α induced injury via suppression of NF-kB p65 mediated MLCK-P-MLC signaling pathway.

2. Materials and methods

2.1. Cell culture

Caco-2 cells were purchased from ATCC (American Type Culture Collection, USA) and maintained at 37 °C in a culture medium composed of Dulbecco's modified Eagle's medium with 4.5 mg/mL glucose, 50 U/mL penicillin, 50 U/mL streptomycin, 25 mmol/L HEPES, and 10% fetal bovine serum as previously described [11,12]. For growth on filters, high density cells (1×10^5 cells) were plated on Transwell filters with 0.4 μm pore size (Corning Incorporated, USA). The medium involving different concentrations of 1,25(OH)2D3 (Sigma–Aldrich, USA) with or without 10 ng/ml TNF-α (Sino biological inc. China) was added to the basolateral compartments of Transwells.

2.2. Trans-epithelial electrical resistance (TEER) measurements

A 12-well transwell system was used for this assay as described previously [13]. Briefly, Caco-2 cells were seeded in the apical

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chamber and the changes of TEER were measured with an epithelial voltohmmeter ERS-2 (Merck Millipore, USA) as described previously [14,15]. About 3 weeks after confluence when the filter-grown Caco-2 monolayers reached epithelial resistance of at least 500 Ω cm^2 [16], the cells were incubated in different reagents as indicated. Electrical resistance was measured until similar values were recorded on three consecutive measurements. Values were corrected for background resistance due to the membrane insert and calculated as Ω cm^2.

2.3. Paracellular marker FD-40 (FITC-Dextran 40 kda) flux measurements

Paracellular permeability was assessed following a previously mentioned method [17,18]. Caco-2 monolayers were treated as described above. After treatment, cells were rinsed with PBS and incubated in the upper chamber with Hank's balanced salt solution containing 1 mg/mL FD-40 solution for 2 h. FD-40 flux was assessed by taking 100uL from the basolateral chamber. Fluorescent signal was measured with Synergy H_2 microplate reader (Biotek Instruments, USA) using 492 nm excitation and 520 nm emission filters. FD-40 concentrations were determined using standard curves generated by serial dilution of FD-40.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total cellular RNA was extracted using the TRIzol one-step extraction method (Trizol reagent; Invitrogen, USA) and reverse transcribed into cDNA using kit (Promega, USA). QRT-PCR was carried out using SYBR PCR master kit (Applied biosystems, USA). Primers were listed 5′–3′ as follows: MLCK: F, CAACAGGGTCACCAACCAGC; R, GCCTTGCAGGTGTACTTGGC; VDR: F, ACT GTC ATT GGG GCT ATC ATC; R, CAG CAG AAT CGC ATC AGG TC; GAPDH: F, GCA CCG TCA AGG CTG AGA AC; R, ATG GTG GTG AAG ACG CCA GT. For each sample, real-time PCR reactions were performed in triplicate. RNA relative expression was calculated as fold change using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$) with GAPDH used as the internal control gene. The relative expression of mRNA in the treated samples was determined as a fold increase compared with control samples.

2.5. Western blot analysis

The whole protein of the filter grown Caco-2 monolayers was extracted using the method as described previously [11]. The concentration of proteins of Caco-2 monolayers was determined using BCA method (Thermo Scientific, USA) and the extracts containing equal quantities of proteins (25 μg) were electrophoresed in 6% or 10% polyacrylamide gel. Subsequently, the separated proteins were transferred onto a PVDF membrane. The membrane was blocked for non-specific binding for 1 h (5% BSA in TBS-Tween 20 buffer) at room temperature, and then incubated overnight at 4 °C with rabbit anti-ZO-1 monoclonal antibody (1:1000 dilution, Invitrogen, USA), rabbit anti-Claudin-1 monoclonal antibody (1:1000 dilution, CST, USA), mouse anti-Occludin monoclonal antibody (1:1000 dilution, Invitrogen, USA), rabbit anti-MLCK monoclonal antibody (1:1000 dilution, Abcam, UK), rabbit anti-MLC antibody(1:1000 dilution, CST, USA), rabbit anti-p-MLC antibody (1:1000 dilution, CST, USA), rabbit anti-VDR monoclonal antibody (1:1000, Abcam, UK) and rabbit anti-GAPDH monoclonal antibody (1:1000 dilution, CST, USA). The membrane was subsequently incubated at room temperature for 1 h with secondary antibodies. Blots were developed with ECL detection reagents (Merck Millipore, USA).

2.6. Immunofluorescent (IF) of ZO-1, Occludin and NF-kB p65 in Caco-2 monolayers

Cell monolayers were treated as indicated above. Cellular localization of the TJ protein ZO-1, Occludin and NF-kB p65 was assessed by immunofluorescence as described previously [11]. Briefly, filters were rinsed with PBS and fixed overnight in 100% methanol at −20 °C and subsequently fixed by 100% acetone at −20 °C for 1 min. Filters were then rinsed in PBS followed by blocking with 1% bovine serum albumin (BSA) for 2 h at room temperature. Filters were incubated with 6 μg/mL anti-rabbit ZO-1 (Invitrogen, USA) and 4 μg/mL anti-mouse Occludin (Invitrogen, USA) or 4ug/ml anti-rabbit NF-kB p65 (CST, USA) antibody overnight at 4 °C. After being washed with PBS, filters were incubated with goat anti-rabbit IgG conjugated to Alexa488 (Molecular Probes, USA) and goat anti-mouse IgG conjugated to Alexa555 (Molecular Probes, USA) in 1% BSA for 1 h at room temperature. After being washed with PBS, cells were mounted using the Prolong Gold anti-fade reagent (Molecular Probes, USA) and stored at 4 °C in dark until analyzed. The fluorescence was visualized under Fluoview 1000 confocal microscope (Olympus, Japan).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared according to the manufacturer's instructions (Thermo Pierce, USA). Briefly, 5×10^6 cells were washed with PBS and resuspended in cytoplasmic extraction reagent and centrifuged for 5 min at 16,000 × g. The supernatant (cytosolic extract) was removed and the pellet resuspended in nuclear extraction reagent. Nuclear extracts were obtained by centrifugation at 16,000 × g for 10 min. The nuclear protein extracts were kept frozen at −80 °C at a concentration of 1 mg/ml until used. DNA–protein binding assays were carried out by DIG Gel Shift Kit (Roche, Germany). Double stranded complementary oligonucleotides containing the NF-kB p65 binding sites were synthesized and end-labeled with DIG: 5′-AGTTGAGGGGACTTTCCCAGGC -3′. Binding reactions were carried out at room temperature in binding buffer using 30 fmol DIG end-labeled target DNA and 5 μg of nuclear extract. Competition assays were performed by adding 125-fold excess of unlabeled probe before the labeled probe. Assays were electrophoresed onto native 8% polyacrylamide gels and transferred onto a positively charged nylon membrane. Transferred DNAs were cross-linked to the membrane at 1200 × 100 μJ/cm^2 and detected using anti-digoxigenin-AP and the chemiluminescent substrate CSPD.

2.8. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and analyzed using a Student *t* tests for unpaired data and ANOVA to compare groups whenever required (GraphPad Prism version 5.0 for Windows; GraphPad Software, USA). A P value <0.05 was used to indicate statistical significance. All experiments were repeated at least three times to ensure reproducibility.

3. Results

3.1. 1,25(OH)2D3 preserves intestinal epithelial barrier function from TNF-α induced injury

Both TEER and FD-40 flux were tested to evaluate monolayer permeability. After being exposed to 10 ng/ml TNF-α for 48 h, the TEER dropped to more or less 50% relative to the baseline ($p < 0.05$)

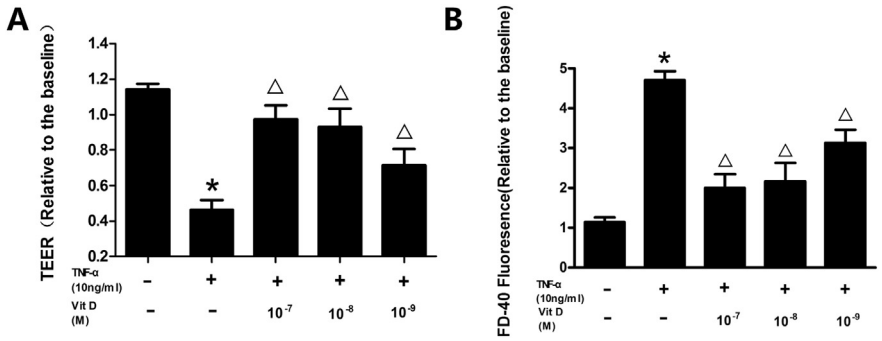

Fig. 1. 1,25(OH)2D3 attenuates intestinal epithelial barrier dysfunction induced by TNF-α. A. Caco-2 monolayers were incubated with or without 10 ng/ml TNF-α in the presence or absence of 10^{-7},10^{-8} or 10^{-9} M 1,25(OH)2D3 for 48 h. 1,25(OH)2D3 significantly attenuated TEER reduction induced by TNF-α treatment. B. Caco-2 monolayers were treated as described in panel a. The TNF-α-induced increase of FD-40 flux was significantly attenuated by 1,25(OH)2D3 treatment. Results were expressed as mean ± SEM (n = 3). *P < 0.05, vs control. △P < 0.05 vs TNF-α.

(Fig. 1A). Consistent with the changes of TEER mentioned above, the flux of FD-40 of monolayers challenged with TNF-α for 48 h was significantly higher relative to the baseline ($p < 0.05$) (Fig. 2B), indicating that the paracellular permeability to nonionic macromolecules was also increased by TNF-α treatment. In order to test the protective effect of 1,25(OH)2D3, increasing doses of 1,25(OH)2D3 were added simultaneously with 10 ng/ml TNF-α to Caco-2 monolayers for 48 h. As shown in Fig. 2, 1,25(OH)2D3 ameliorated both the drop of TEER and the increase of FD-40 flux induced by TNF-α in a dose-dependent way and 1,25(OH)2D3 at 10^{-7} M produced the strongest effect ($p < 0.05$).

3.2. 1,25(OH)2D3 ameliorates TNF-α-induced altered localization of ZO-1 and Occludin

Both the decrease of TJ protein expression level and the alteration of TJ protein localization are believed to be involved in the injury of intestinal epithelial barrier function induced by multiple reagents [14,19]. Thus, we examined the effect of 1,25(OH)2D3 and TNF-α on the expression levels of TJ proteins in Caco-2 monolayers treated with or without TNF-α in the absence or presence of 1,25(OH)2D3. As shown in Fig. 2, the expression levels of TJ proteins including ZO-1, Occludin and Claudin-1 were not significantly

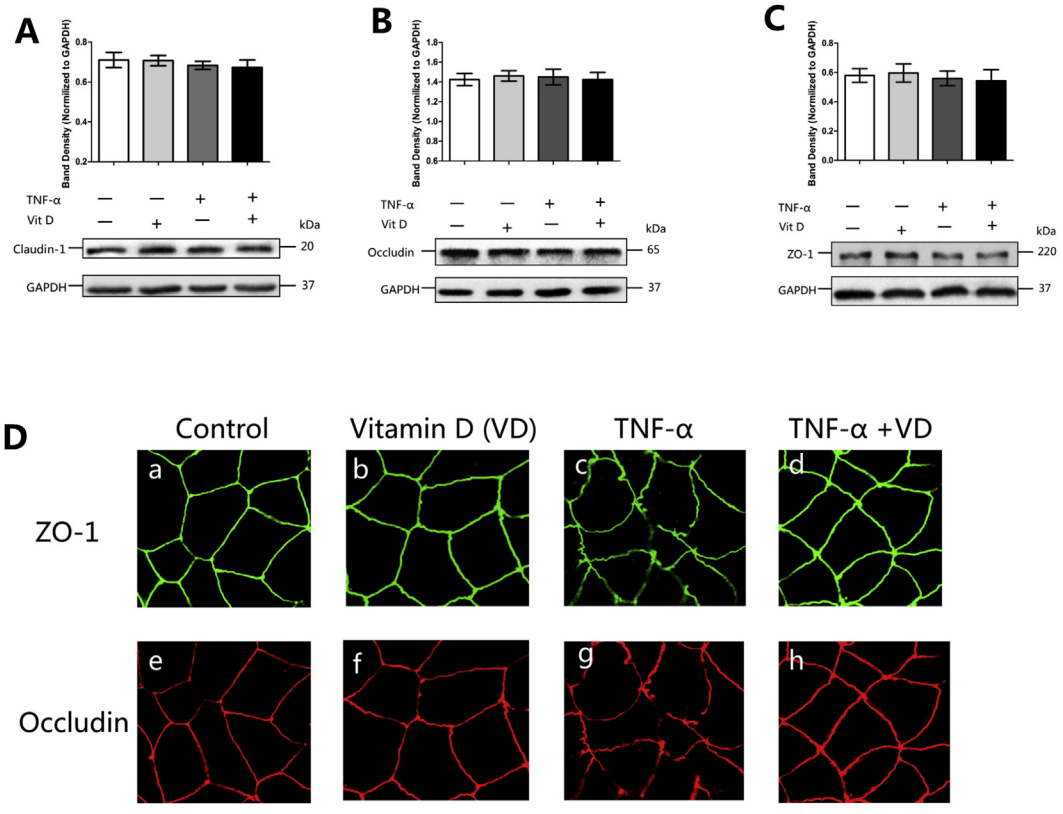

Fig. 2. The effect of 1,25(OH)2D3 and TNF-α on the expression level and localization of TJ proteins. (A, Claudin-1; B, Occludin; C, ZO-1). Caco-2 monolayers were treated as described in Fig. 1 and the total protein was harvested after treatment for 48 h for western blotting assays. Results were expressed as mean ± SEM (n = 3). D, Caco-2 monolayers were incubated without or with 10 ng/ml TNF-α in the absence or presence of 10^{-7} M 1,25(OH)2D3 for 48 h. TJ proteins ZO-1, Occludin were stained by immunofluorescence. 1,25(OH)2D3 dramatically prevented the TNF-α-induced morphological disruption of tight junction proteins ZO-1 and Occludin in Caco-2 monolayers.

altered by the treatment with or without TNF-α in the absence or presence of 1,25(OH)2D3. This result suggested that the alteration of TJ distribution instead of the decrease of TJ protein expression levels might be the mechanism underlying the change of monolayer permeability induced by TNF-α.

In this study, ZO-1 and Occludin were chosen as markers of the localization of TJ proteins. In normal Caco-2 monolayers, immunofluorescence of ZO-1 and Occludin showed smooth edges and a typical "chicken wire" shape (Fig. 2D,a,e). However, after being exposed to 10 ng/ml of TNF-α for 48 h, the cellular edges became serrated and changes were observed at sites of cell to cell contact, the ZO-1 network pattern was disrupted with areas of irregular labeling intensity, some part hardly continuous (Fig. 2D,c). Alterations in Occludin localization were similar to that of ZO-1 (Fig. 2D,g). These results suggested that TNF-α caused abnormal distribution of these TJ proteins. However, co-treatment with 10^{-7} M 1,25(OH)2D3 ameliorated TNF-α-induced altered localization of ZO-1 and Occludin, immunofluorescence remained typical "chicken wire" like in the TNF-α+VD groups (Fig. 2D,d,h).

3.3. Effect of 1,25(OH)2D3 on TNF-α-induced activation of MLCK-P-MLC signaling pathway

It is widely reported that MLCK-P-MLC pathway plays a pivotal role in the physiological and pathophysiological regulation of the localization of TJ proteins [20–22]. Based on the above-mentioned remarkable protective effect of 1,25(OH)2D3 on TNF-α induced altered localization of TJ proteins, the effect of 1,25(OH)2D3 on TNF-α-induced activation of MLCK-P-MLC signaling pathway was further investigated. The protein of monolayers were harvested after 48 h treatment with or without 10 ng/ml TNF-α in the absence or presence of 10^{-7} M 1,25(OH)2D3 and the expression level of MLCK and phosphorylation level of MLC were tested by western blotting. As demonstrated in Fig. 3A and B, after treatment of Caco-2 monolayers with TNF-α for 48 h, there was a significant increase in both the expression of MLCK and phosphorylation level of MLC compared with control ($P < 0.05$). However, 1,25(OH)2D3 co-treatment significantly inhibited the increase of both the expression of MLCK and the phosphorylation level of MLC compared with Caco-2 monolayers treated with TNF-α ($P < 0.05$). This result suggested that 1,25(OH)2D3 attenuated TNF-α-induced intestinal barrier dysfunction by suppressing the MLCK-P-MLC signaling pathway.

3.4. 1,25(OH)2D3 ameliorates TNF-α-induced injury of intestinal epithelial barrier function by blocking NF-kB p65 signaling pathway

NF-kB p65 signaling is believed to mediate TNF-α induced up-regulation of the expression level of MLCK [23,24]. Recently, 1,25(OH)2D3 was shown to inhibit the NF-kB p65 activation by VDR signaling pathway [9,25]. Based on our findings mentioned above, we hypothesized that 1,25(OH)2D3 inhibited TNF-α-induced activation of MLCK-P-MLC signaling pathway by blocking the activation of NF-kB p65.

The nuclear translocation and activation of NF-kB p65 was tested by EMSA and immunofluorescence. As shown in Fig. 3C and D, 1,25(OH)2D3 alone had no effect on the activation of NF-kB p65. TNF-α, as expected, caused nuclear translocation and activation of NF-kB p65. However, pre-incubating Caco-2 monolayers with 1,25(OH)2D3 for 48 h alleviated the activation of NF-kB p65 activation induced by TNF-α. Immunofluorescence of NF-kB p65 showed that treatment of Caco-2 monolayers with 10 ng/ml TNF-α for 30 min elicited NF-kB p65 accumulation within the nucleus. Pre-incubation with 10^{-7} M 1,25(OH)2D3 for 48 h decreased the nuclear accumulation of NF-kB p65 induced by TNF-α. These findings suggest that the protective effect of 1,25(OH)2D3 on TNF-α-induced injury of intestinal epithelial barrier function is produced mainly by blocking the activation of NF-kB p65 signaling.

3.5. Effect of 1,25(OH)2D3 on TNF-α induced down-regulation of VDR expression

To test the hypothesis that VDR signaling plays a part in the protective effect of 1,25(OH)2D3, the mRNA and protein levels of VDR in Caco-2 monolayers were tested after treatment with or without 10 ng/ml TNF-α in the absence or presence of 10^{-7} M 1,25(OH)2D3 for 48 h. As shown in Fig. 4A and B, TNF-α inhibited the expression of VDR significantly at both mRNA and protein level ($P < 0.05$). 1,25(OH)2D3, as expected, up-regulated the expression of VDR and largely reversed the inhibition induced by TNF-α($P < 0.05$).

4. Discussion

The injury of intestinal epithelial barrier function is found to play a pivotal role in multiple enteropathies. During the initiation and development of intestinal inflammation, plethora of inflammatory cytokines within the mucosa including TNF-α is commonly seen [26]. Plethora of studies have demonstrated that inflammatory cytokines play important roles in disrupting intestinal epithelial barrier function [4,7,24].

Both the decreased expression and the altered localization TJ proteins are found to be related with the injury of TJ function induced by inflammatory cytokines [21,22,27]. Here in, our work suggested that exposing Caco-2 monolayers to 10 ng/ml TNF-α for 48 h didn't affect the expression level of TJ proteins. The results of immunofluorescence suggested that the altered localization of TJ proteins played pivotal role in TNF-α induced injury of intestinal epithelial barrier function.

MLCK-P-MLC signaling pathway was firstly reported by Turner et al. to regulate barrier function by remodeling the localization of TJ proteins [23]. Following studies suggested that MLCK-P-MLC signaling pathway was an important pathway in regulating the intestinal barrier function in multiple physiological and pathological situations [28–30]. Based on the results of immunofluorescence, we tested the expression level of MLCK and the phosphorylation level of MLC by immunoblotting. The results suggested that TNF-α upregulated the expression of MLCK and phosphorylation of MLC significantly, indicating the involvement of MLCK-P-MLC signaling pathway in TNF-α induced intestinal barrier dysfunction in Caco-2 monolayers, which was also in coincidence with previous reports [26,27].

1,25(OH)2D3, also termed as calcitrol, was reported to protect barrier function in both the endothelial and epithelial cells [9,31]. Based on the previous reports, we set out to investigate the possible protective effect of 1,25(OH)2D3 on TNF-α induced barrier dysfunction and the underlying mechanisms. The results suggested that 1,25(OH)2D3 significantly attenuated the TNF-α induced decrease of TEER and increase of FD-40 flux. The altered localization of TJ proteins induced by TNF-α was also ameliorated by 1,25(OH)2D3. The effect of 1,25(OH)2D3 on TNF-α elicited activation of MLCK-P-MLC signaling pathway was then tested. The results showed that adding 1,25(OH)2D3 with TNF-α at baseline together significantly attenuated TNF-α induced activation of MLCK-P-MLC signaling pathway.

Actually, it is believed that the anti-inflammatory effect of 1,25(OH)2D3 is mainly produced by VDR signaling, which functions as an important pathway in modulating the activation of NF-kB p65 signaling [9,27,32]. Additionally, the increased expression of MLCK induced by TNF-α was also reported to be mediated mainly by

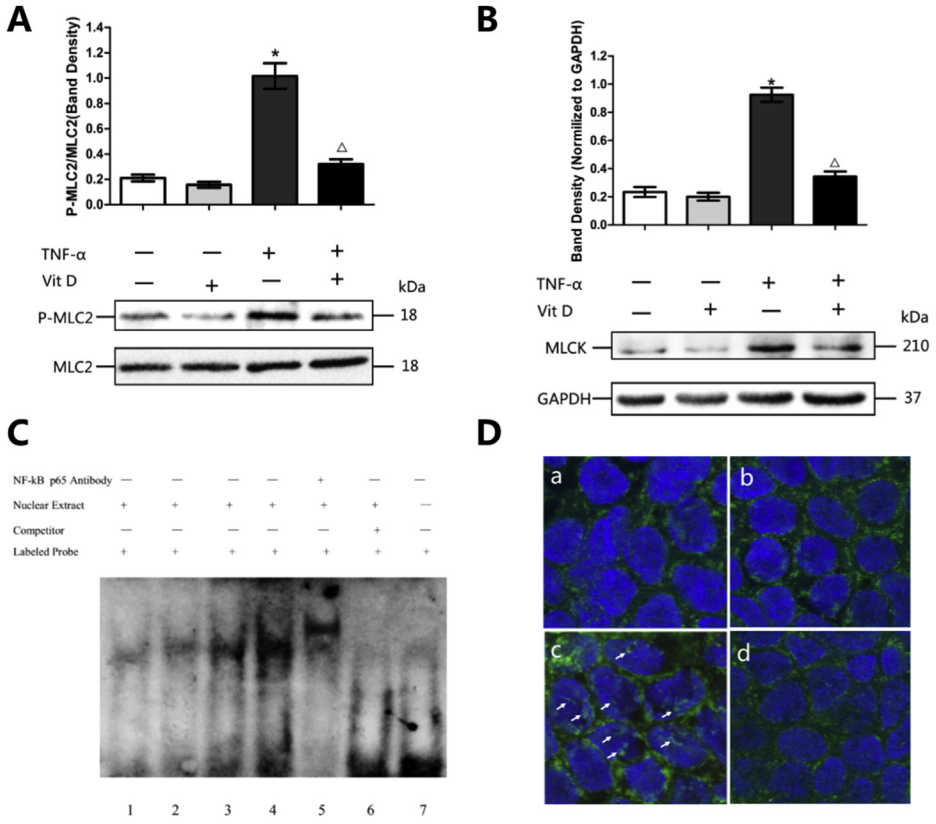

Fig. 3. 1,25(OH)2D3 inhibited TNF-α induced increased phosphorylation of MLC2 and expression of MLCK by blocking NF-kB p65 signaling. A,B, The total protein of monolayers were harvested after incubation for 48 h in the absence or presence of 10 ng/ml TNF-α with or without 10^{-7} M 1,25(OH) 2 D3 followed by western blotting assays. Results were expressed as mean ± SEM (n = 3). *P < 0.05, vs control. △P < 0.05 vs TNF-α. C, 1,Nuclear extracts from monolayers without treatment with TNF-α or 1,25(OH)2D3 served as control; 2, Nuclear extracts from monolayers treated with 10^{-7} M 1,25(OH)2D3 for 48 h in the absence of TNF-α; 3, Nuclear extracts from monolayers pre-treated with 10^{-7} M 1,25(OH)2D3 for 48 h followed by exposing to 10 ng/ml TNF-α for 30 min; 4, Nuclear extracts from monolayers exposed to 10 ng/ml TNF-α for 30 min; 5, NF-kB p65 super-shift: NF-kB p65 monoclonal antibody was added with the nuclear extracts harvested from the monolayers treated with 10 ng/ml TNF-α for 30 min; 6, Cold-Probe was added with the nuclear extracts harvested from the monolayers treated with 10 ng/ml TNF-α for 30 min to rule out nonspecific conjugation of the labeled probe; 7, Negative control without nuclear extract. D, Caco-2 monolayers were stained for NF-kB p65 by immunofluorescence. Pre-treatment with 10^{-7} M 1,25(OH)2D3 for 48 h significantly inhibited the nuclear translocation of NF-kB p65 elicited by TNF-α. a: Control; b: Caco-2 monolayers were incubated with 10^{-7} M 1,25(OH)2D3 for 48 h without exposing to TNF-α; c: Caco-2 monolayers were exposed to 10 ng/ml TNF-α for 30 min; d: Caco-2 monolayers were pre-incubated with 10^{-7} M 1,25(OH)2D3 for 48 h followed by exposing to 10 ng/ml TNF-α for 30 min(Arrows were added to direct against distinct changes).

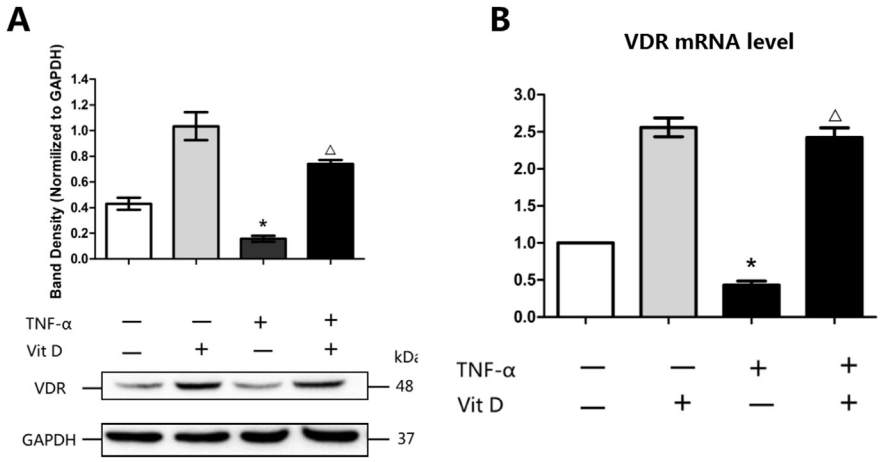

Fig. 4. 1,25(OH)2D3 reversed TNF-α induced down-regulation of VDR at both protein and mRNA level. The total protein or RNA of monolayers were harvested after incubation for 48 h in the absence or presence of 10 ng/ml TNF-α with or without 10^{-7} M 1,25(OH) 2 D3. The protein and mRNA level of RNA were tested by western blotting (A) and qRT-PCR (B) respectively. Results were expressed as mean ± SEM (n = 3). *P < 0.05, vs control. △P < 0.05 vs TNF-α.

NF-kB p65 signaling [26,33]. In this study, the involvement of VDR signaling and NF-kB pathway was further tested by immunoblotting, EMSA and immunofluorescence. The results suggested that 1,25(OH)2D3 significantly reversed the TNF-α induced decrease of VDR expression. TNF-α, as expected, caused activation and nuclear translocation of NF-kB p65 as validated by EMSA and immunofluorescence. 1,25(OH)2D3, as expected, inhibited the TNF-α elicited activation of NF-kB p65 signaling.

In conclusion, this study shows that 1,25(OH)2D3 preserves intestinal epithelial barrier function from TNF-α induced injury via suppression of NF-kB p65 mediated MLCK-P-MLC signaling pathway. Since MLCK-P-MLC signaling pathway is widely involved in the regulation of TJ function in multiple physiological and pathological situations. Our work suggests that VDR signaling might be a potential target for developing new therapy for multiple TJ related diseases.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.125.

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