


Angiopietin-2 silence alleviates lipopolysaccharide-induced inflammation, barrier dysfunction and endoplasmic reticulum stress of intestinal epithelial cells by blocking Notch signaling pathway

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ABSTRACT

Necrotizing enterocolitis, a devastating gastrointestinal disease with high mortality, poses great threats to global health. Therefore, we conducted this study to explore the role of ANGPT2, as well as the potential mechanism, in necrotizing enterocolitis. IEC-6 cells were stimulated with lipopolysaccharide (LPS) to induce necrotizing enterocolitis model in vitro. The expression of ANGPT2 was measured by RT-qPCR. The cell viability was detected using CCK-8. Besides, the expressions of endoplasmic reticulum (ER) stress-related proteins, Notch signaling pathway-related proteins and tight junction proteins were checked by western blot. The apoptosis and inflammatory response were detected by TUNEL and ELISA, respectively. Moreover, with the adoption of TEER, the cell monolayer permeability was detected. The results showed that ANGPT2 expression was greatly increased after LPS induction. In addition, ANGPT2 knockdown significantly decreased the apoptosis, inflammatory response, barrier dysfunction and endoplasmic reticulum stress of LPS-induced IEC-6 cells. What is more, ANGPT2 knockdown could block Notch signaling pathway. Additionally, with the treatment of Jagged-1, the protective effect of ANGPT2 knockdown on LPS-induced intestinal injury was partly abolished. To sum up, silencing ANGPT2 could improve LPS-induced inflammation, barrier dysfunction and ER stress of intestinal epithelial cells via blocking Notch signaling pathway.

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Introduction

Necrotizing enterocolitis (NEC), an acute inflammatory disease of intestine, predominantly affects preterm infants and contributes to higher morbidity, mortality as well as cost [1,2]. It is found that 10% preterm infants whose birth weight is lower than 1,500 g are vulnerability to NEC [3]. The common complications of NEC are presented as neurodevelopmental delay, failure to thrive, gastrointestinal problems as well as cholestasis [2]. Although many researchers have spared no efforts to study NEC in recent years, the pathogenesis of NEC still remains obscure and the methods for prevention and treatment are in shortage [4,5]. Therefore, it is urgently required to explore the mechanism of NEC as well as seek for effective therapies for the improvement of NEC.

Angiopietin-2 (ANGPT2), a member of major angiogenic growth factors, is a partial Tie2 antagonist or agonist that regulates Angpt1/Tie2 signaling in context-dependent [6] and organ-specific manners

[7,8]. The role of ANGPT2 in many diseases has been investigated. For instance, the expression of ANGPT2 was found to be increased in lung cancer tissues, while ANGPT2 inhibition could improve lung adenocarcinoma to some degree [9]. It was also found that ANGPT2 inhibition helped to promote ischemic cardiovascular remodeling, thus alleviating ischemic heart failure [10]. Moreover, Zhilin Li et al. reported that the inhibition of ANGPT2 could improve the integrity of blood-brain barrier and decreased the expressions of inflammatory cytokines [11]. Of note, ANGPT2 expression was testified to be upregulated in patients living with Crohn's disease [12], indicating a potential link between ANGPT2 and intestine-related diseases; however, whether ANGPT2 is involved in NEC still remains unclear.

As a highly evolutionarily conserved network, Notch orchestrates cell fate determination and participates in the proliferation, migration, differentiation and cell death of organisms [13]. It was found that the inhibition of Notch signaling pathway could protect

the integrity of intestinal permeability against damage [14]. Besides, the intestinal integrity and colitis could be improved by Notch signaling pathway inhibition [15]. What is more, Gao et al. testified that Notch-1 could be induced by ANGPT2 in vascular endothelial cell, suggesting Notch signaling might be a downstream pathway in response to ANGPT2 [16]. Furthermore, endoplasmic reticulum stress (ERs), a multifunctional intracellular organelle that contributes to the synthesis and folding of proteins, calcium storage and signaling, is an important factor that participates in the development of NEC [17,18].

Therefore, we speculated that ANGPT2 knock-down could improve NEC via modulating Notch signaling pathway. This study aims to explore the role of ANGPT2 in NEC cell model, as well as the underlying mechanism of action.

Material and methods

Cell culture, treatment and transfection

Intestinal epithelial IEC-6 cells that obtained from Cell Bank of the Chinese Academy of Sciences were cultured in Dubelcco's modified eagle medium (DMEM; Gibco, NY, USA) with 10% fetal bovine serum (FBS; Gibco, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; CA, USA) at 37°C with 5% CO₂ in a humidified incubator. Subsequently, the cells were treated with different concentrations of lipopolysaccharide (LPS, 0, 0.1, 1, 10, and 100 µg/ml) for 24 h.

To knock down the expression of ANGPT2 in IEC-6 cells, short hairpin RNA (sh-RNA) specific to ANGPT2 (sh-ANGPT2-1 and sh-ANGPT2-2) as well as corresponding negative control (sh-NC) were generated by Genscript Biotech (Nanjing, China). For transfection, IEC-6 cells were inoculated into 24-well plates. Upon reaching 80% confluency, the transfection was operated with the application of Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA).

CCK-8

Intestinal epithelial IEC-6 cells were seeded into 96-well plates and incubated with different concentrations of LPS for 24 h. Then, each well was added with 10 µL CCK-8 reagent (Beyotime,

Shanghai, China) to incubate the cells for additional 4 h at 37°C. The optical density was detected by a microplate reader (BioTek, Vermont, USA) under the condition of $\lambda = 450$ nm.

RT-qPCR

Total mRNA from IEC-6 cells was isolated with the help of Trizol reagents (Invitrogen, NY, USA). Then, extracted RNA was synthesized to complementary DNA (cDNA) through PrimeScript™ RT Reagent Kit (TaKaRa, Tokyo, Japan). SYBR Green RT-qPCR Master Mix kit (Takara, Tokyo, Japan) was adopted to perform real-time quantitative PCR on ABI 7500 quantitative PCR instrument (Applied Biosystems, CA, USA). The relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method [19].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Cell apoptosis of IEC-6 cells was detected with the application of TUNEL assay kits (Invitrogen; Thermo Fisher Scientific) in line with manufacturer's specification. IEC-6 cells got fixed with 4% paraformaldehyde for 15 min and then permeabilized in 0.25% Triton-X 100 for 20 min at room temperature. After that, the cells were incubated with TUNEL reaction solution for 1 h according to manufacturer's protocol. After staining with 4',6-diamidino-2-phenylindole (DAPI) for 30 min, IEC-6 cells were under observation by an inverted microscope (magnification; Olympus Corporation).

Enzyme-linked immunosorbent assay (ELISA)

The release of inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and prostaglandin E₂ (PGE₂), was measured by ELISA kits (Beyotime, Shanghai, China). With the adoption of a microplate reader (Bio-Rad, USA), the optical density at 450 nm was recorded.

Transepithelial Electrical Resistance Measurements (TEER)

In order to detect the cell monolayer permeability of IEC-6 cells, TEER was measured with the help of a millicell-ERS apparatus (Millipore, USA) [20].

TEER was recorded 30 min after changing medium at room temperature, thus ensuring temperature equilibration and uniformity of culture environment. Collagen coated Transwell inserts without cells were used for the correction of background resistance.

Western blot

Total proteins from IEC-6 cells were extracted by RIPA lysis buffer (Beyotime Institute of Biotechnology) and the quantification of these proteins was conducted using BCA kits (Thermo Fisher Scientific, Inc.). Exposed to 10% SDS-PAGE, the proteins were then transferred onto PVDF membranes. After the procedure of impeding membranes with 5% nonfat milk and incubating with corresponding primary antibodies against ANGPT2 (Abcam, ab155106), barrier dysfunction-related proteins (ZO-1 (Abcam, ab221547), occludin (Abcam, ab167161), claudin-1 (Biorbyt, orb10447)), ERs-related proteins (GRP79 (Abcam, ab21685), CHOP (Biorbyt, orb314857), p-PERK (Biorbyt, orb474911), PERK (Biorbyt, orb338886)), Notch signaling-related proteins (Notch1 intracellular domain (NICD1) (Abcam, ab167441), HES1 (Abcam, ab108937)), and GAPDH (Abcam, ab9485) at 4°C overnight, secondary antibodies were applied to incubate membranes for 2 h. Finally, the protein bands were photographed using enhanced chemiluminescence (ECL).

Statistical analysis

Data collected from experiments were presented as mean \pm standard deviation. The statistical significance of differences among groups was analyzed with the application of one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons. Value <0.05 was viewed to be statistically significant.

Results

The expression of ANGPT2 was increased in IEC-6 cells after LPS induction

First, to induce NEC model in vitro, IEC-6 cells were stimulated by different concentrations of LPS

(0, 0.1, 1, 10, and 100 μ g/ml) for 24 h. As Figure 1a shows, the cell viability was decreased in LPS-induced IEC-6 cells, and the effects of LPS induction on IEC-6 cells were in a concentration-dependent manner. Moreover, the mRNA expression and protein expression of ANGPT2 was increased by LPS induction (Figure 1b and Figure 1c), especially at 10 μ g/ml. Thus, LPS at 10 μ g/ml was used for subsequent experiments.

ANGPT2 knockdown inhibited the apoptosis of LPS-induced IEC-6 cells

Subsequently, to explore the role of ANGPT2 in LPS-induced IEC-6 cells, IEC-6 cells were transfected with sh-ANGPT2-1 or sh-ANGPT2-2 to knock down ANGPT2. Evidently, the expression of ANGPT2 in IEC-6 cells, no matter at mRNA level or protein level, was significantly decreased after transfection (Figure 2a and Figure 2b). Due to the higher efficiency of knockdown, sh-ANGPT2-1 was used for subsequent experiments. Then, the transfected or untransfected IEC-6 cell were stimulated by LPS. According to Figure 2c, the decreased cell viability induced by LPS was enhanced by ANGPT2 knockdown in comparison with LPS+sh-NC. Additionally, the increased apoptosis level in LPS-induced IEC-6 cells was decreased after knocking down ANGPT2 (Figure 2d).

ANGPT2 knockdown inhibited the inflammatory response, barrier dysfunction and ER stress of LPS-induced IEC-6 cells

Next, a series of detection experiments were performed to explore the role of ANGPT2 in LPS-induced intestinal injury. Just as Figure 3a–Figure 3d demonstrated, the expressions of proinflammatory cytokines in IEC-6 cells, including PGE2, TNF- α , IL-1 β and IL-6, were greatly increased by LPS induction, while ANGPT2 silence suppressed the promotive effects of LPS induction on inflammatory response. Besides, TEER experiments were conducted to measure the cell permeability, and the smaller the resistance value, the higher the permeability. As shown in Figure 3e, the decreased resistance value of LPS-induced IEC-6 cells was upregulated by ANGPT2 knockdown,

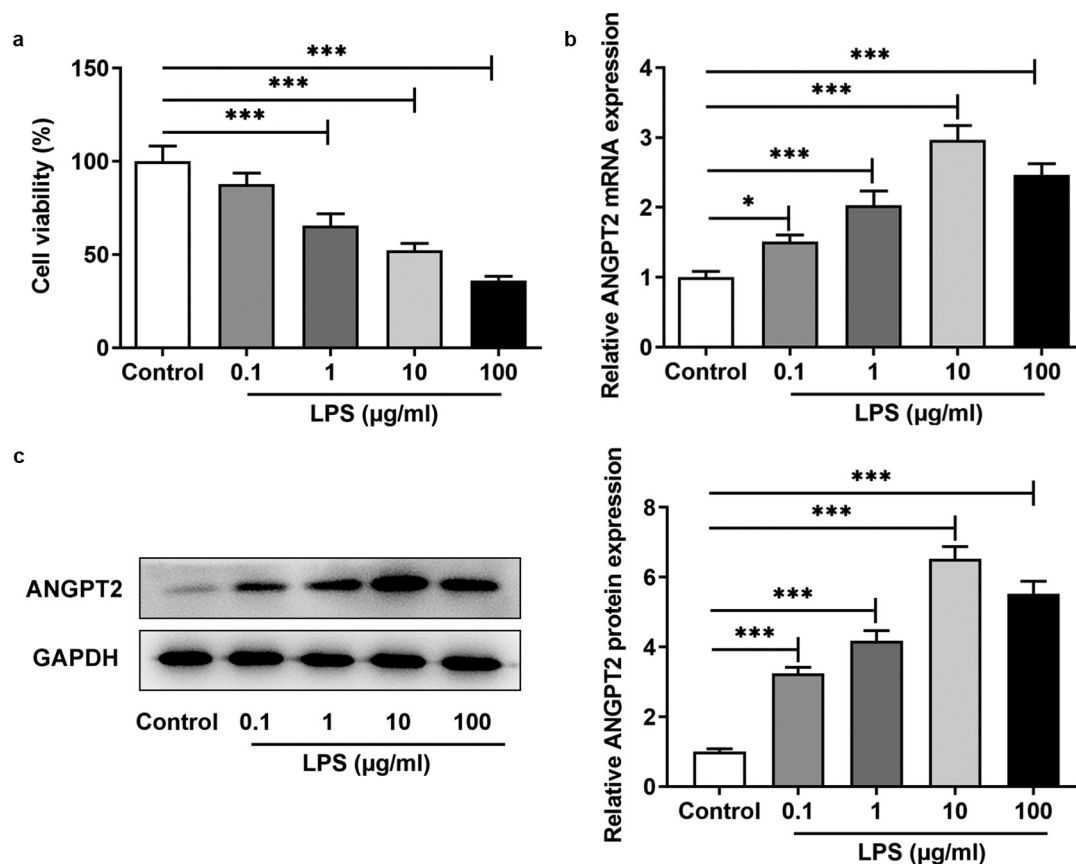


Figure 1. The expression of ANGPT2 was increased in IEC-6 cells after LPS induction. (a) IEC cells were incubated with different concentrations of LPS (0, 0.1, 1, 10, and 100 μg/ml) for 24 h. The cell viability of LPS-induced IEC-6 cells was detected using CCK-8. (b) The mRNA level of ANGPT2 in LPS-induced IEC-6 cells was detected using RT-qPCR. (c) The protein expression of ANGPT2 in LPS-induced IEC-6 cells was detected using western blot. * $p < 0.05$ and *** $p < 0.001$ vs Control.

suggesting that the high permeability induced by LPS in IEC-6 cells was alleviated by ANGPT2 knockdown. In addition, compared with Control, the expressions of ZO-1, occludin and claudin-1 were decreased by LPS induction, while ANGPT2 knockdown increased those decreased expressions (Figure 3f). What is more, results from Figure 3g revealed that ANGPT2 knockdown downregulated the expressions of GRP79, CHOP and p-PERK in IEC-6 cells, but upregulated PERK expression.

ANGPT2 knockdown blocked Notch signaling pathway

To further explain the regulatory mechanism, the critical proteins of Notch signaling were evaluated by western blot. As Figure 4 displays, the expressions of Notch1 intracellular domain (NICD1) and hairy and enhancer of split-1 (HES1) gained a huge growth in IEC-6 cells after LPS induction

in comparison with Control. Nevertheless, the increased expressions of NICD1 and HES1 were decreased after knocking down ANGPT2, revealing that ANGPT2 knockdown could block Notch signaling pathway.

ANGPT2 knockdown inhibited the apoptosis of LPS-induced IEC-6 cells through suppressing Notch signaling pathway

To further ensure the crucial role of Notch signaling underlying the regulatory role of ANGPT2 in LPS-induced intestinal injury, Jagged-1 (JAG), an agonist of Notch, was applied for treatment. Results from Figure 5a indicate that the decreased cell viability of LPS-induced IEC-6 cells was hugely enhanced by ANGPT2 knockdown, while JAG reversed the promotive effects of ANGPT2 knockdown on LPS-induced IEC-6 cells. It was also found that the apoptosis level was increased after

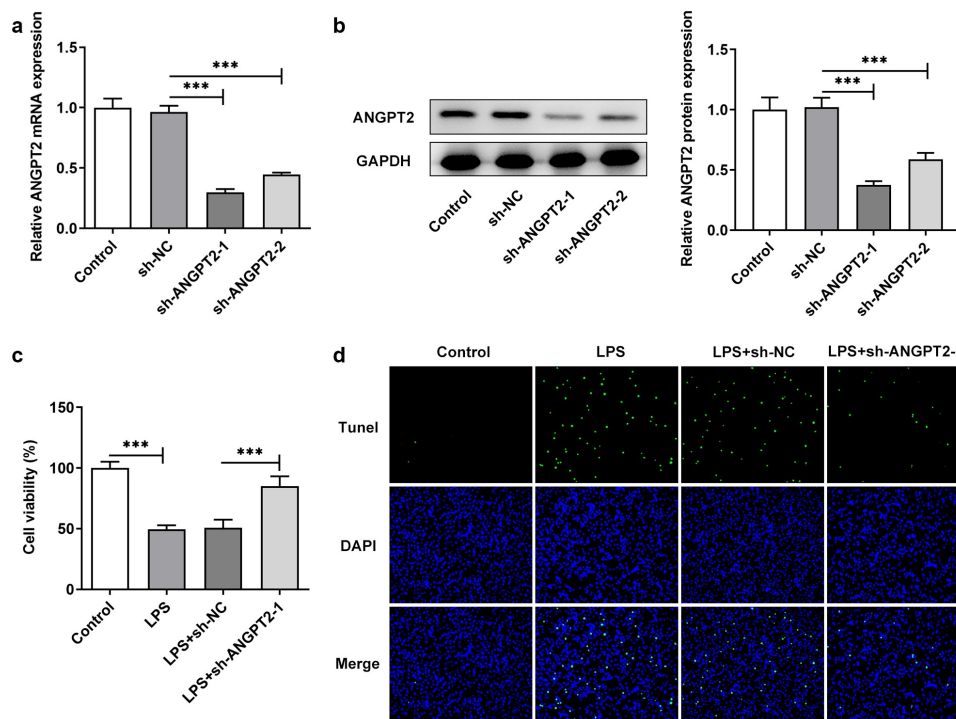


Figure 2. ANGPT2 knockdown inhibited the apoptosis of LPS-induced IEC-6 cells. (a) IEC-6 cells were transfected with sh-NC or sh-ANGPT2-1/2, and the mRNA expression and (b) the protein expression of ANGPT2 in IEC-6 cells were detected by RT-qPCR and western blot, respectively. (c) The transfected or untransfected IEC-6 cells were induced with LPS (10 μ g/ml) for 24 h. The cell viability of was detected using CCK-8. (d) The cell apoptosis of each group was checked by TUNEL. *** $p < 0.001$.

the treatment of JAG in contrast with LPS+sh-ANGPT2-1 (Figure 5b).

ANGPT2 knockdown improved the inflammatory response, barrier dysfunction and ER stress of LPS-induced IEC-6 cells via suppressing Notch signaling pathway

Furthermore, according to ELISA, the increased levels of PGE₂, TNF- α , IL-1 β and IL-6 in LPS-induced IEC-6 cells were greatly decreased after knocking down ANGPT2, while JAG reversed the inhibitory effects of ANGPT2 knockdown on LPS-induced IEC-6 cells (Figure 6a–Figure 6d). Compared with LPS+sh-ANGPT2-1, the decreased cell permeability was elevated after the treatment of JAG (Figure 6e). Besides, the expressions of ZO-1, occludin and claudin-1 in LPS-induced IEC-6 cells were increased by ANGPT2 knockdown, while JAG reversed the promotive effects of ANGPT2 knockdown on LPS-induced IEC-6 cells, revealing that ANGPT2 knockdown could improve barrier dysfunction (figure 6f). Moreover, the treatment of JAG upregulated the

expressions of GRP79, CHOP and p-PERK but downregulated PERK expression compared with LPS+sh-ANGPT2-1 (Figure 6g).

Discussion

Necrotizing enterocolitis, one of the most common and life-threatening diseases, has an incidence of 3–15%, which varies among geographical area [21]. In recent years, numerous studies have been conducted; however, no study investigated the mechanism of NEC from the angle of ANGPT2. This study firstly testified that ANGPT2 inhibition could be an effective therapeutic therapy to improve NEC. In the present study, we found that the expression of ANGPT2 was increased in LPS-induced IEC-6 cells, revealing that ANGPT2 might be involved in NEC. It was also found that the inhibition of ANGPT2 could suppress the apoptosis, inflammatory response, barrier dysfunction and ER stress of LPS-induced IEC-6 cells. More importantly, ANGPT2 inhibition improves NEC via blocking Notch signaling pathway.

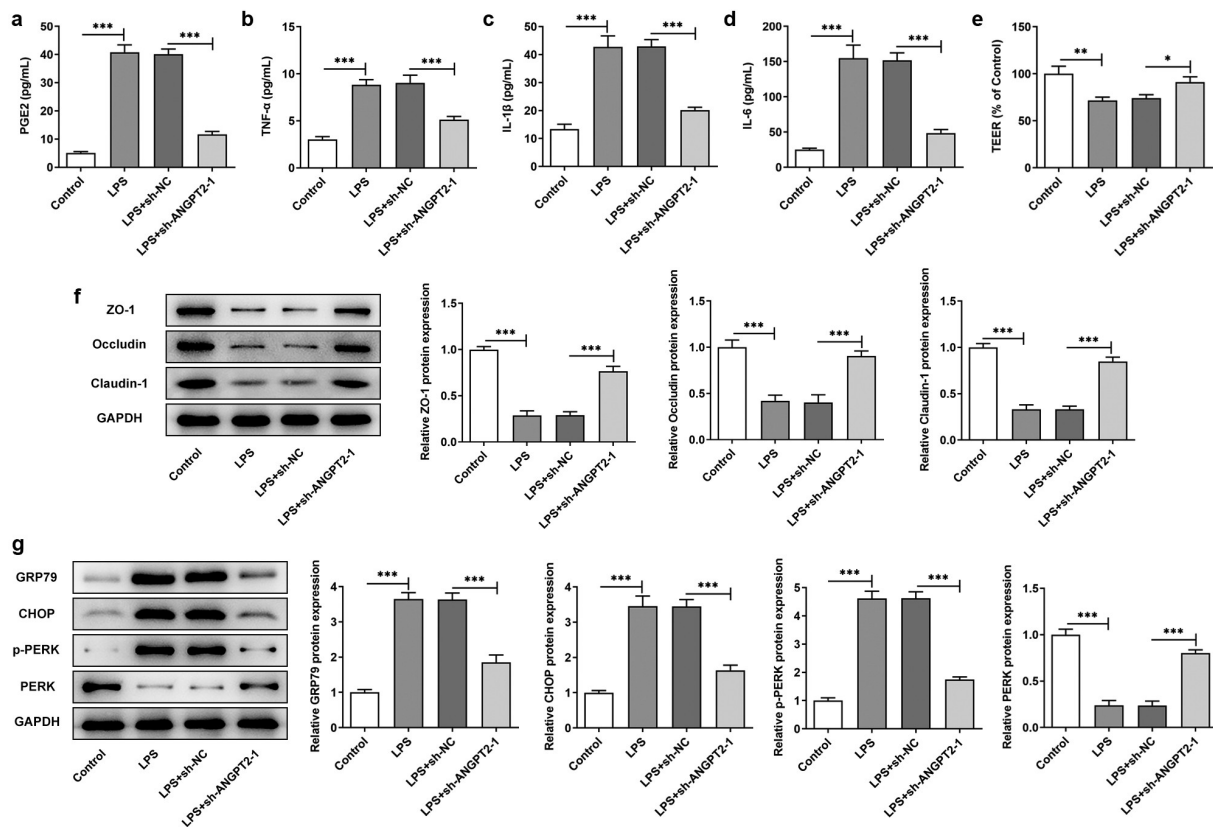


Figure 3. ANGPT2 knockdown inhibited the inflammatory response, barrier dysfunction and ER stress of LPS-induced IEC-6 cells. (a–d) The transfected or untransfected IEC-6 cells were induced with LPS (10 μ g/ml) for 24 h. The levels of PGE2, TNF- α , IL-1 β and IL-6 were checked by ELISA. (e) The cell monolayer permeability was detected using TEER. (f) The expressions of ZO-1, occludin and claudin-1 were measured by western blot. (g) The expressions of GRP79, CHOP, p-PERK and PERK were measured by western blot. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

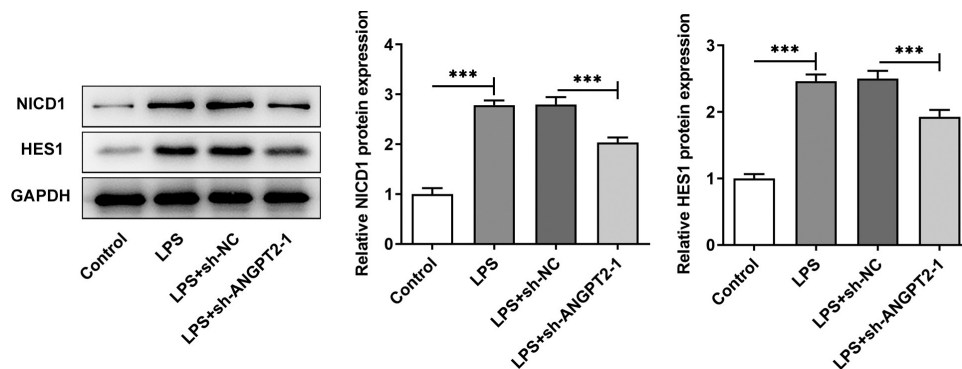


Figure 4. ANGPT2 knockdown blocked Notch signaling pathway. The transfected or untransfected IEC-6 cells were induced with LPS (10 μ g/ml) for 24 h. The expressions of NICD1 and HES1 were measured using western blot. *** $p < 0.001$.

ANGPT2, a vascular disruptive agent with antagonistic activity, acted as an important player in physiological processes and presented deregulated expression in several diseases [22]. A previous study verified a close connection and regulatory relationship between ANGPT2 and Notch signaling [16,23]. In the present study, we

found that ANGPT2 knockdown blocked Notch signaling pathway, evidenced by the decreased expressions of NICD1 and HES1. To further confirm this finding, we adopted JAG, an agonist of Notch, to treat LPS-induced IEC-6 cells, discovering that JAG reversed the inhibitory effects of ANGPT2 knockdown on inflammatory response,

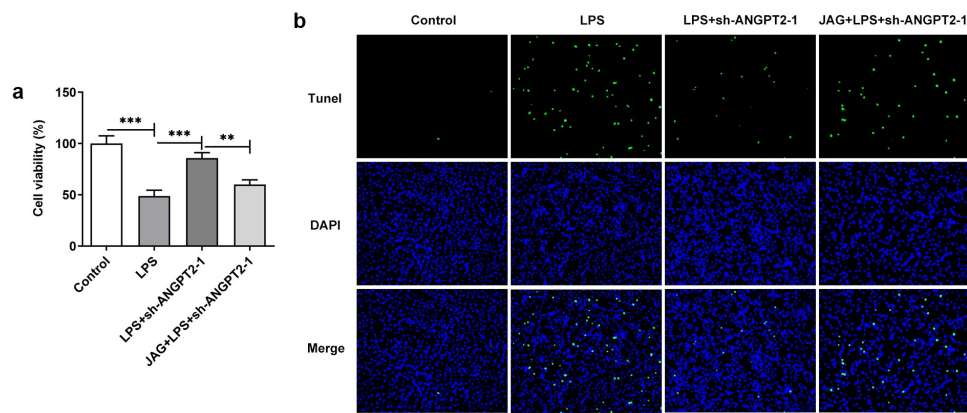


Figure 5. ANGPT2 knockdown inhibited the apoptosis of LPS-induced IEC-6 cells through suppressing Notch signaling pathway. (a) The transfected or untransfected IEC-6 cells were induced with LPS (10 $\mu\text{g}/\text{ml}$) for 24 h. Jagged-1 (JAG), an agonist of Notch, was used for treatment. The cell viability was detected using CCK-8. (b) The cell apoptosis of each group was checked using TUNEL. $**p < 0.01$, $***p < 0.001$.

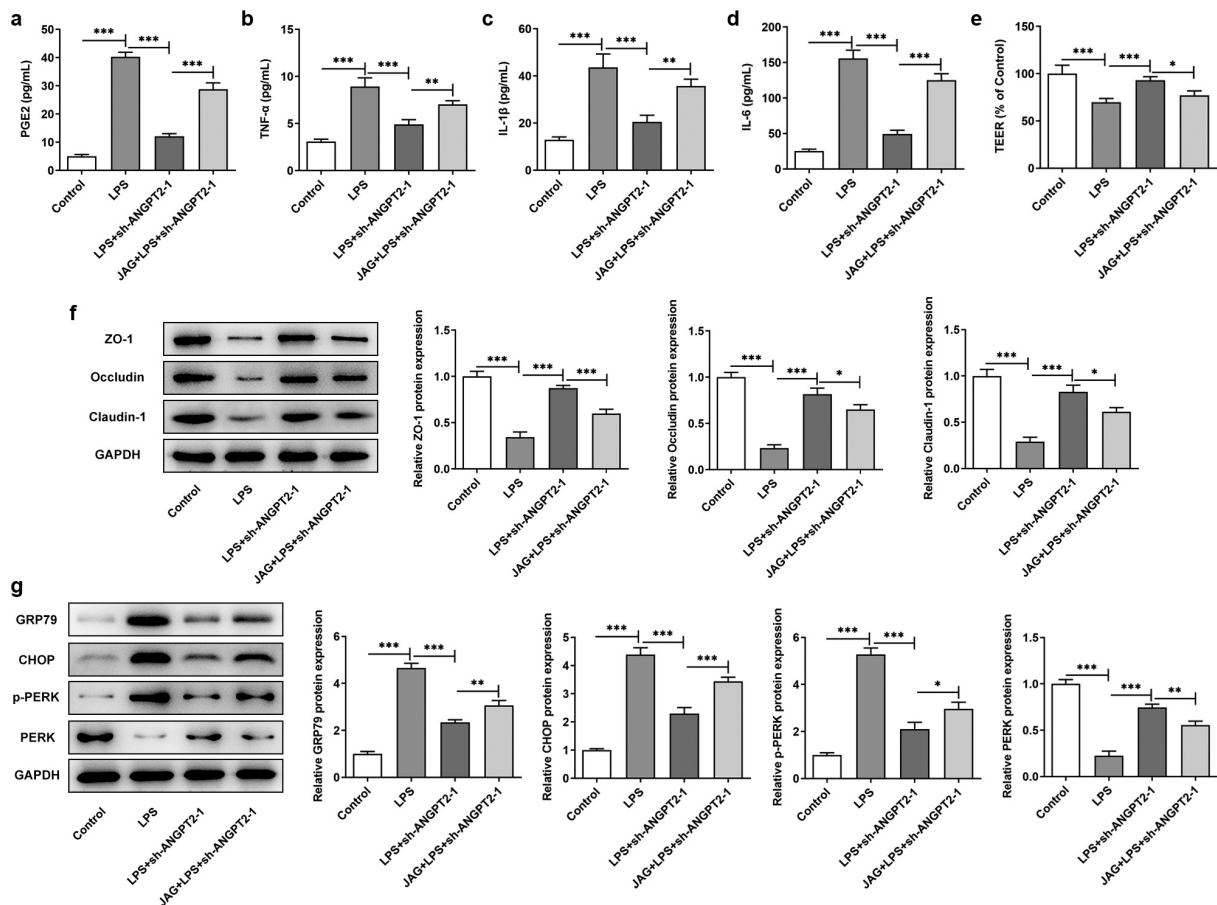


Figure 6. ANGPT2 knockdown improved the inflammatory response, barrier dysfunction and ER stress of LPS-induced IEC-6 cells via suppressing Notch signaling pathway. (a–d) The transfected or untransfected IEC-6 cells were induced with LPS (10 $\mu\text{g}/\text{ml}$) for 24 h. Jagged-1 (JAG), an agonist of Notch, was used for treatment. The levels of PGE2, TNF- α , IL-1 β and IL-6 were checked by ELISA. (e) The cell monolayer permeability was detected using TEER. (f) The expressions of ZO-1, occludin and claudin-1 were measured by western blot. (g) The expressions of GRP79, CHOP, p-PERK and PERK were measured by western blot. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

barrier dysfunction and endoplasmic reticulum stress in LPS-induced IEC-6 cells, which implied that ANGPT2 knockdown exerted its inhibitory effects via regulating Notch signaling pathway.

Inflammatory response and barrier dysfunction are important pathogenesis of NEC. NEC is characterized by inflammation, and persistent inflammation can result in impaired intestinal barrier, allowing for bacterial translocation and promote the progression of disease [24,25]. It has been reported that overexpression of Sirtuin-1 relieves NEC by reducing the inflammatory response and intestinal epithelial barrier dysfunction [26]. Zhang D et al. disclosed that milk fat globule membrane could alleviate NEC by suppressing LPS-induced inflammatory response in IEC-6 cells [27]. Coincidentally, ANGPT2 played an important role in inflammatory response and barrier dysfunction. Fiedler U et al. declared that ANGPT2 acted as an autocrine regulator in endothelial cell inflammatory responses and regulated the activities of proinflammatory cytokines [28]. The high levels of ANGPT2 exacerbated inflammatory cell and cytokine infiltration in LPS-induced lung injury [29]. In addition, a previous study testified that ANGPT2 was closely related with the destabilization of the endothelium, thus resulting in intestinal barrier dysfunction in acute pancreatitis [30]. Results obtained from this study revealed that the increased expressions of PGE₂, TNF- α , IL-1 β and IL-6 in LPS-induced IEC-6 cells were decreased by ANGPT2 knockdown, and the cell monolayer permeability in LPS-induced IEC-6 cells, as well as the expression of tight junction proteins, was decreased by ANGPT2 knockdown, revealing that ANGPT2 silence exerted inhibitory effects on inflammatory response and barrier dysfunction in NEC.

Furthermore, ERs is an important factor in necrotizing enterocolitis. Xiaoli Zhu reported that ERs, which had a close relationship with intestinal inflammation, acted as a crucial player in the pathogenesis of NEC [17]. Evidence also showed that ERs participated in the development of NEC [31]. In the present study, we discovered that the expressions of GRP79, CHOP and p-PERK in LPS-induced IEC-6 cells were decreased by ANGPT2 silence through inhibiting Notch signaling pathway.

Conclusion

To sum up, ANGPT2 silence inhibited the apoptosis, inflammatory response, barrier dysfunction and endoplasmic reticulum stress in LPS-induced IEC-6 cells, while JAG reversed the inhibitory effects of ANGPT2 silence, revealing that ANGPT2 knockdown improved NEC via blocking Notch signaling pathway.

Data availability statement

All data in this study have been included in this article.

Disclosure statement

The authors have no conflicts to declare.

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