Phosphatidylcholine regulates NF-κB activation in attenuation of LPS-induced inflammation: evidence from in vitro study

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ABSTRACT

Phosphatidylcholine (PC) has been demonstrated as anti-inflammatory and antioxidant/pro-oxidant molecules. In this study, we investigated the protective effects of PC on inflammatory bowel disease (IBD) caused by lipopolysaccharide (LPS)-induced injury in intestinal epithelia cells. The IEC-6 cells (intestinal epithelia cells) were stimulated with LPS (1 μg/mL) for 24 h with or without PC pretreatment, in the next steps: (1) the level of the inflammatory cytokine tumor necrosis factor (TNF-α) was measured with ELISA; (2) the nuclear translocation and phosphorylation of NF-κB was investigated with Western blot, EMSA, immunofluorescence assay; (3) the protein phosphorylation levels in MAPK signaling pathway were detected with Western blot method. The results showed: (1) compared with the normal group, 10 and 20 μg/mL of PC significantly inhibited the production and activation of TNF-α, (P < 0.01); (2) pretreatment with PC inhibited LPS-induced nuclear translocation and phosphorylation of p65 in IEC-6 cells; (3) pretreatment with PC inhibited the protein phosphorylation levels in MAPK signaling pathway. Our findings indicated that PC had the effect to protect IEC-6 cells from LPS-induced injury and this effect was exerted possibly through inhibiting the TNF-α secretion, down-regulating nuclear translocation and phosphorylation of p65 and inhibiting MAPK signaling pathways.

1. Introduction

The inflammatory bowel disease (IBD) is a group of chronic inflammatory conditions of the gastrointestinal tract, which comprises Crohn disease (CD) and ulcerative colitis (UC) and whose incidence and prevalence is increasing worldwide (Cosnes et al. 2011). Although the pathogenesis of IBD is still unclear, it is thought to arise from a combination of multiple factors including genetic influences, alterations in the gut microbiota, alterations in the innate and adaptive immune system and environmental exposures.

The commensal bacterial component, such as lipopolysaccharide (LPS), may cause dysregulation of the intestinal mucosal immune response and this could be a main etiology of IBD (Maloy and Powrie 2011). Because the gut-microbial homeostasis mainly depends on the immune response of tissue-resident macrophage and the barrier function of intestinal epithelial cells (IECs) (Smith et al. 2011), the bacterial translocation across the intestinal barrier and the subsequent activation of macrophage by LPS have been believed to play a critical role in the dysregulation of intestinal mucosal immune response (Peterson and Artis 2014). The key regulator of immune response of intestinal mucosa is thought to be the nuclear transcription factor-κB (NF-κB) (Atreya et al. 2008), since NF-κB activation was generally founded in mucosal macrophage and colonic epithelial cells from IBD patients (Neurath et al. 1996). Furthermore, the agents blocking NF-κB signaling pathway was reported as a new therapeutic strategy for IBD (Kim and Jobin 2005).

The mechanisms that protect gastrointestinal (GI) mucosa against potential damage resulting from luminal constituents include blood flow to remove noxious elements, secretion of fluids to neutralize luminal acid or inactivate enzymes, presence of anti-biotic or healing peptides, strengthening of tight junctions between cells, and presence of a mucous gel layer to coat the surface and impede uptake of damaging agents (Allen and Flemstrom 2005). The mucus gel itself is overlaid with a coating of surface-active phospholipid, primarily phosphatidylcholine (PC, Figure 1), which creates a hydrophobic nature to the surface, particularly in the stomach and colon (Hills...
et al. 1983). A number of studies have shown that pathological states or therapeutic agents that cause gastric and intestinal diseases can also lead to a reduction in GI surface hydrophobic properties (Lichtenberger et al. 2006; Qin et al. 2008). Thus, maintenance of the PC, which is majorly constituting the hydrophobic surface, can be important for physical health and prevention of disease.

In previous studies, it was found: (1) PC was able to protect experimental rats from a number of ulcerogenic agents and/or conditions including nonsteroidal anti-inflammatory drugs (NSAIDs), which are chemically associated with PC; moreover, pretreating a number of the NSAIDs with exogenous PC prevented a decrease in the hydrophobic characteristics of the mucus gel layer and protected rats against the injurious GI side effects of NSAIDs while enhancing and/or maintaining their therapeutic activity (Lim et al. 2013); (2) in the cell model for ulcerative colitis, the several processes of the model cell caco-2, including activation of both MAPKs ERK and p38 and transport of NF-kappaB subunits to the nucleus, were significantly inhibited by exogenous addition of PC (Treede et al. 2007); (3) oral PC could preserve the gastrointestinal mucosal barrier during LPS-induced inflammation (Dial et al. 2008); [4] docosahexaenoic acid could attenuate LPS-stimulated inflammatory response by regulating the PPARγ/NF-kB pathways in primary bovine mammary epithelial cells (He et al. 2017). However, there was currently no report to simultaneously study the protective effect of PC on LPS-induced inflammation and the molecular mechanism of this effect and establish their relation involving NF-kB. In this study, the protective effect and the inner mechanism involving NF-kB of PC on the LPS-induced inflammation was studied and the relation between those two was established, which was the novelty of this investigation.

2. Methods and materials

2.1. Cell culturing procedures

The non-transformed rat small intestine epithelial cell line IEC-6 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS, PAA, Toronto, Canada), 100 mg/mL insulin (Invitrogen, Grand Island, NY), and 10 mL/L penicillin-streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 10% CO₂. Cultured cells were digested with 0.25% trypsin (Invitrogen) when they reached 85%-90% confluence, followed by re-suspension in fresh medium. Cell concentration was adjusted to 9 × 10⁵ cells/L and cultured for another 48 h.

2.2. Cell viability assay

The cytoprotective activity of PC against LPS-induced injury was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, IEC-6 cells were seeded into 96-well plates at a density of 1.0 × 10⁴/mL and allowed to adhere overnight. After incubation, cells were pretreated with or without PC (provide by National Institutes for Food and Drug Control of China) at a series of concentrations (2.5, 5.0, 10.0, or 20.0 μg/mL) for 1 h before exposure to LPS (Escherichia coli 055: B5, Sigma; used at 1.0 μg/mL) for 24 h. After cell treatment, 20.0 μL of 5.0 mg/mL MTT solution was added to each well, followed by incubation at 37°C for 4 h. The resultant formazan crystals were then dissolved in 150.0 μL DMSO, and absorbance was measured at 490 nm using an EnSpire Multimode Plate Reader. The cytotoxicity of PC was measured by exposing IEC-6 cells to 2.5, 5.0, 10.0, or 20.0 μg/mL of PC for 24 h. The cell viability was measured using the MTT method as described above.

2.3. Quantification of pro-inflammatory cytokine secretion

The secretion of TNF-α from the IEC-6 cell was quantified with ELISA kits purchased from Shanghai Jijin Chemistry Technology and Diaclone (Shanghai, China). Briefly, IEC-6 cells were seeded into 24-well plates at 5.0 × 10⁴ cells/well and cultured overnight. The cells were treated with escalating concentrations of PC (2.5, 5.0, 10.0, and 20.0 μg/mL) for 1 h before exposure to LPS for 24 h. After cell stimulation, cell-free supernatants were collected for the determination of TNF-α production according to the manufacturer’s instructions.
2.4. Western blot analysis

The IEC-6 cells (1 x 10^7 cells) were cultured in 10 cm-dishes and allowed to adhere for 24 h. After being pre-treated with or without PC at concentrations of 5, 10 and 20 μg/mL and exposure to LPS for 24 h, cells were collected and lysed with lysis buffer containing protease inhibitors (Beyotime Institute of Biotech, Haimen, Jiangsu, China). The cytosolic fraction and the nuclear fraction were separated. Proteins from both fractions were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich, MO, USA) and incubated overnight at 4°C with primary polyclonal antibodies against 1-κBα (1:200 diluted, Santa Cruz Biotechnology, Inc., CA, USA), p-1-κBα (1:500, Sigma-Aldrich), NF-κB-p65 (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA), p-NF-κB-p65 (1:800, CST), JNK (1:1000, Abcam, Cambridge, CA, USA), p-JNK, ERK (1:1000, Abcam), p-ERK (1:100, Santa Cruz Biotech, Inc), p38 (1:1000, Abcam), p-p38 (1:100 diluted, Santa Cruz Biotech, Inc), or β-actin (1:300, Santa Cruz Biotech, Inc), followed by rinsing for 3 times with PBST for 15 min, and subsequently incubated at room temperature for 1 h with peroxidase-conjugated secondary goat anti-rabbit antibody (1:2000, Beijing Solarbio science & technology Co., Ltd) or goat anti-mouse IgG (1:2000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The membrane was washed with PBST for 3 times (15 min per time) and then visualized using SuperSignal West Pico ECL reagents (Pierce, ThermoFisher Scientific Inc., Waltham, MA, USA). Lab Works Image Acquisition and Analysis Software version 4.5 was used to quantify band intensities.

2.5. Electrophoretic mobility shift assay

To further determine NF-κB activity, we performed an electrophoretic mobility shift assay (EMSA). In brief, nuclear extracts of IEC-6 cells stimulated with LPS and intervened with PC were prepared and incubated with 32P-end-labeled 43-mer double-stranded oligonucleotide (15 μg protein with 16 fmol DNA), for 30 min at 37°C. The sequence of the double-stranded oligonucleotide was: 5'-TTGTTACAA AGT TGA GGG GAC TTT CCC AGG C AGGGAGGCGTCG-3' (boldface indicates NF-κB binding sites). Then, the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The nuclear extract from the IEC-6 cells without intervention with LPS and PC was used as the negative control. The dried gel was visualized with a Storm 820 scanner (Amer sham Biosciences, USA).

2.6. Immunofluorescence analysis

Immunofluorescence staining was carried out to detect the nuclear translocation of NF-κB protein in the IEC-6 cells. Briefly, IEC-6 cells were seeded into 12-well culture plates at 1.0 x 10^5 cells/well and incubated overnight. Cells were treated with or without PC at concentrations of 5, 10 or 20 μg/mL before exposure to LPS for 24 h. For immunofluorescent staining, cells on the chamber slides were washed 3 times with phosphate-buffered saline (PBS, 10 min per time), fixed with 4% (v/v) paraformaldehyde at room temperature for 30 min, and permeabilized with PBS containing 0.1% TritonX-100 for 5 min at RT. Afterwards, the samples were washed 3 times with PBS (10 min per time), followed by blocking with PBS containing 5% bovine serum albumin (BSA) for 1 h at RT. Cells were then incubated overnight at 4°C with polyclonal rabbit anti-rat NF-κB p65 antibody (1:100, CST), washed 3 times with PBS, and then incubated with Cy3-labeled goat anti-rabbit IgG (1:500, Beyotime) for 2 h at RT. Fluorescence images were captured using a microscope. The cells were then incubated with 4', 6-diamidino-2-phenylindole (DAPI, 1.5 μg/ml) for 20 min at room temperature in the dark and rinsed 3 times with PBS. Slides were mounted in Vectashield mounting medium and fluorescent signals were examined under a fluorescence microscope. The fluorescence intensity in individual stained cells was analyzed using the Image-pro Plus 7 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.7. Statistical analysis

Each experiment was performed at least in triplicate. All data were presented as the mean ± standard deviation (SD). Significance between various treatment samples was calculated One-Way analysis of variance (ANOVA), followed by Turkey's post-hoc multiple comparisons test. Differences between the mean values were evaluated with a minimal significance of P < 0.05. All statistical analyses were carried out using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. The effect of PC on the viability of IEC-6 cells

First we evaluated the potential effects of PC on the viability of small intestine epithelial cells with the MTT assay after IEC-6 cells were treated with PC at a series of
concentrations (0, 5, 10, or 20 μg/mL) for 24 h. As shown in Figure 2, there were no apparent cytotoxic effects on the growth of IEC-6 cells when incubated with PC.

3.2. Pc suppressed the production of inflammatory cytokines in LPS-treated IEC-6 cells

To analyze the potential anti-inflammatory properties of PC, the production of pro-inflammatory cytokines were examined in LPS-stimulated IEC-6 cells. As shown in Figure 3, incitement with LPS resulted in a significant increase in the production of TNF-α, compared with the unstimulated control (P < 0.05). However, pretreatment with PC led to a dose-dependent inhibition of cytokine secretion. It was noteworthy that 10 and 20 μg/mL of PC considerably decreased the production of TNF-α, compared with the cells in absence of PC pretreatment (P < 0.01).

3.3. Pc inhibited the activation of NF-κB in LPS-incited IEC-6 cells

The nuclear factor-κB (NF-κB) is a category of transcription factors that play crucial roles in mediating pro-inflammatory response to pathogenic stimulation. Here we examined whether PC (0, 5, 10, and 20 μg/mL) could restrain the LPS-induced NF-κB activation, including nuclear translocation and phosphorylation, and this was associated with degradation of I-κBα protein that regulated NF-κB activity in IEC-6 cells. The patterns of I-κBα, NF-κB-p65, p-I-κBα, and p-NF-κB-p65 were detected in the cytosol and nucleus sections respectively by Western blot assay. As shown in Figure 4(A), the protein level of I-κBα increased in the cytosol of LPS-treated cells along with the increase of intervention concentration of PC, which indicated that PC could induce the expression of I-κBα, and the protein level of NF-κB-p65 decreased in the nucleus of LPS-treated cells along with the increase of intervention concentration of PC, which indicated that PC could inhibit the translocation of NF-κB-p65 into nucleus; meanwhile, as shown in Figure 4(B), the level of p-I-κBα decreased in the cytosol of LPS-treated cells along with the increase of intervention concentration of PC, which indicated that PC could inhibit the activation of I-κBα, and the level of p-NF-κB-p65 decreased in the nucleus of LPS-treated cells along with the increase of intervention concentration of PC, which indicated that PC could inhibit the phosphorylation of NF-κB-p65 in the nucleus. In summary, the above results demonstrated that PC could inhibit the activation of the NF-κB pathway upon LPS stimulation of IEC-6 cells.

3.4. Pc inhibited the activity of NF-κB in the nucleus of LPS-incited IEC-6 cells

To detect the effect of PC on the activity of NF-κB in the nucleus of LPS-incited IEC-6 cells, EMSA was performed. As shown in Figure 5, the strength of the signal from the NF-κB-DNA complex decreased along with the increase of the intervention concentration of PC. The
Figure 4. PC suppressed the activation (translocation and phosphorylation) of NF-κB in LPS-stimulated IEC-6 cells. Cells were pretreated with PC (0, 5, 10, 20 μg/mL) for 1 h, and stimulated with LPS (1 μg/mL) for 24 h. Western blot was performed to determine the proteins in nuclear or cytosolic fractions by using anti-rat I-κBα, p65, p-I-κBα and p-p65 antibodies. β-actin was used as the internal reference. (A) Detection of I-κBα, p65; (B) detection of p-I-κBα, p-p65.

Figure 5. PC suppressed the NF-κB activity inside the nucleus of NF-κB in LPS-stimulated IEC-6 cells. Cells were pretreated with PC (0, 5, 10, 20 μg/mL) for 1 h, and stimulated with LPS (1 μg/mL) for 24 h. EMSA was performed to determine the NF-κB activity in nuclear fraction by using DNA probe specific to NF-κB.
result demonstrated that PC could inhibit the activity of NF-κB in the nucleus of LPS-incited IEC-6 cells.

3.6. Pc inhibited the translocation of NF-κB to nucleus

Immunofluorescence staining was exploited to detect the nuclear translocation of p65, the respective subunit of NF-κB. We found that p65 was tethered to the cytoplasm in absence of LPS stimulation, whereas nuclear p65 was markedly aggrandized upon LPS incitement and the existence of p65 in the nucleus of IEC-6 cells incited by LPS gradually disappeared along with the increase of the intervention concentration of PC and finally, at 20 μg/ml PC, almost all of p65 signal existed in the cytoplasm and no p65 existed in the nucleus of IEC-6 cells incited by LPS (Figure 6).

3.7. Pc regulated the LPS-activated phosphorylation of MAPKs

It has been reported that LPS stimulation can activate MAPK signaling pathways, including ERK, JNK and p38, which leads to subsequent phosphorylation and degradation of NF-κB/IκBα complex, and allowed free NF-κB dimmers translocation to nucleus. To delineate the role of MAPK signaling pathway on the fate of NF-κB, we examined the overall expression and phosphorylation status of ERK, JNK and p38 in LPS-stimulated IEC-6 cells. We found that increased phosphorylation of ERK, JNK and p38 caused by 24 h-LPS exposure was impaired by PC pretreatment in a dose-dependent manner (0, 5, 10 or 20 μg/mL). These findings implied that PC-mediated suppression of LPS-induced NF-κB activation through inhibiting MAPK signaling pathways in IEC-6 cells (Figure 7).

4. Discussion

This study, for the first time, demonstrated that the anti-inflammatory effect of PC on the LPS-stimulated rat small intestine epithelial IEC-6 cells was associated with steady

![Figure 6](image) PC suppressed nuclear translocation of NF-κB. Cells were pretreated with PC (0, 5, 10, 20 μg/mL) for 1 h, prior to exposure to 24 h-LPS (1 μg/mL) treatment. Cells were incubated with anti-p65 antibodies, followed by FITC-labeled secondary antibody. The subcellular localization of p65 was visualized using a confocal microscope (630 × magnification).

![Figure 7](image) PC inhibited ERK, JNK and p38 activation. Cells were pretreated with PC (0, 5, 10, 20 μg/mL) for 1 h, and stimulated with LPS (1 μg/mL) for 24 h. Total cell lysates were subjected to Western blot with specific antibodies against p-ERK1/2, p-JNK1/2 and p-p38. Total ERK1/2, JNK1/2, and p38 levels were also assessed, with β-actin used as a loading control.
state of NF-κB through decreasing the TNF-α secretion and activation, inhibiting the translocation of NF-κB and inhibiting the MAPK pathway.

NF-κB is reported to regulate the expression of various genes including cytokines, iNOS, and COX-2, whose activation is responsible for the secretion of pro-inflammatory mediators such as NO, PGE2, TNF-α, and IL-1β (Nolan et al. 2003; Lamkanfi and Dixit 2012; Erler and Monaghan 2015). Under unstimulated conditions NF-κB (p50 and p65 subunits) is sequestered in the cytoplasm and binds to its inhibitory protein I-κB, which prevents NF-κB from entering into the nuclei (Barnes and Karin 1997). Upon treatment, I-κBα undergoes phosphorylation and liberates NF-κB, thus resulting in NF-κB translocation into nucleus where it binds to the specific promoter regions of target genes (Lin and Karin 2007). In previous study, increased nuclear translocation of p65 upon LPS stimulation resulted in the induction of iNOS and COX-2 genes (Chen et al. 1999). It has been shown that redundant p65 in IEC-6 cells elevates the production of inflammatory cytokines, while down-regulation of p65 by overexpressing I-κBα was responsible for the decreased inflammatory cytokines (Lee et al. 2014). Other studies also suggested that suppression of IKK-mediated I-κBα degradation, which usually happens in untreated cells, may also occur in TNF-α-stimulated IEC-6 in the presence of PC (Yang et al. 2015). In our study, LPS-triggered increase in the phosphorylation of NF-κB (p65) was substantially suppressed by PC in a dose-dependent manner. Furthermore, we also found I-κBα degradation was inhibited by PC. Our results basically conformed to the above findings.

Activation of NF-κB was found to be regulated by canonical MAPKs including ERK, JNK, and p38 (Mukherjee et al. 2013). We found that activation of NF-κB upon LPS stimulation was consistent with increased phosphorylation of ERK1/2, JNK1/2 and p38 in IEC-6. However, PC abolished the LPS-induced activation of MAPK signaling pathways, accompanied by a decreased transcriptional activity of NF-κB. The involvement of ERK, JNK and p38 signaling cascades in activation of NF-κB has been indicated in several cell lines, and blockage of these pathways by specific inhibitors inhibited NF-κB activation. The suppressed MAPKs phosphorylation by PC may explain the attenuated NF-κB-mediated inflammatory response upon LPS incitement. Since MAPKs are involved in tumor growth, apoptosis, and inflammation, controlling MAPKs signaling cascades by PC may be considered as a novel strategy to inhibit and manage the inflammatory diseases (Hseu et al. 2015).

Based on the findings made in this study, we drew a schematic diagram on effect of PC in the IEC-6 cells upon LPS incitement (Figure 8).

In conclusion, our study demonstrated: (1) PC held anti-inflammatory and antioxidant effects against LPS stimulation; (2) the mechanism of those anti-inflammatory and antioxidant effects of PC were: (I) decreasing the secretion and activation of TNF-α; (II) inhibiting the translocation and phosphorylation of p65; (III) inhibiting the protein phosphorylation in the MAPK pathway. Our findings established the relationship between the shallow phenomena about the protective effect of PC and the deep molecular mechanism relative to the secretion and activation of TNF-α, the translocation and phosphorylation of NF-κB, and the protein phosphorylation of the MAPK pathway, which were currently innovative in the relative research field.

**Disclosure statement**

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