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The phosphatidylinositol 3-kinase/Akt pathway negatively regulates Nod2-mediated NF-κB pathway

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ABSTRACT

Nucleotide-binding oligomerization domain containing proteins (Nods) are intracellular pattern recognition receptors (PRRs) that recognize conserved moieties of bacterial peptidoglycan and activate downstream signaling pathways, including NF-κB pathway. Here, we show that Nod2 agonist muramyldipeptide (MDP) induces Akt phosphorylation in time and dose-dependent manner. The pharmacological inhibitor of phosphatidylinositol 3-kinase (PI3K) (wortmannin) and dominant-negative forms of p85 (the regulatory subunit of PI3K) or Akt enhance, while constitutive active forms of p110 (the catalytic subunit of PI3K) or Akt inhibit, NF-κB activation and the target gene interleukin (IL)-8 induced by MDP. In addition, the pharmacological inhibitors of PI3K (wortmannin and LY294002) enhance phosphorylation of NF-κB p65 on Ser529 and Ser536 residues, which result in enhanced p65 transactivation activity. Furthermore, we show that the inhibition of PI3K by the pharmacological inhibitors prevent the inactivation of glycogen synthase kinase (GSK)-3β, suggesting that the negative regulation of PI3K/Akt on MDP-induced NF-κB activation is at least in part mediated through inactivation of GSK-3β. Taken together, our results demonstrate that PI3K/Akt pathway is activated by Nod2 agonist MDP and negatively regulates NF-κB pathway downstream of Nod2 activation. Our results suggest that PI3K/Akt pathway may involve in the resolution of inflammatory responses induced by Nod2 activation.

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1. Introduction

Evolution of host defense systems against microbial pathogens is the key for the survival of all multicellular organisms. Transmemberane Toll-like receptors (TLRs) play crucial roles in the detection of invading microbes and the induction of immune and inflammatory responses to defend the host [1,2]. In addition to TLRs, recently identified nucleotide-binding oligomerization domain containing protein family (Nods) recognizes intracellular bacterial products and activates proinflammatory signaling pathways [3,4]. Nod1 and Nod2 protein have been shown to be activated by bacterial components through their leucine-rich repeat domain at the COOH terminus. While the minimal peptidoglycan structure recognized by Nod1 is a dipeptide, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) derived mostly from Gram-negative

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Abbreviations: TLRs, Toll-like receptors; PRR, pattern recognition receptors; LRR, leucine-rich repeat domain; Nod, the nucleotide-binding oligomerization domain family; NF-κB, nuclear factor-κB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PGN, peptidoglycan; LPS, lipopolysaccharide; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid; MDP, muramyldipeptide MurNAc-L-Ala-D-isoGln; GSK-3β, glycogen synthase kinase-3β; RICK, Rip-like interacting CLARP kinase; IKK, IκB kinase; IκB, inhibitor of NF-κB; DN, dominant-negative; CD, Crohn's disease.

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bacteria, the minimal PGN that Nod2 recognizes is the muramyldipeptide, MurNAc-L-Ala-D-isoGln (MDP) from both Gram-positive and Gram-negative bacteria [4]. The activation of Nod2 leads to the recruitment of a CARD-domain containing kinase, RICK/RIP2, through a CARD–CARD interaction, which then leads to the interaction with the regulatory subunit of IKK, IKKγ/NEMO, linking to the activation of NF-κB and proinflammatory gene expression [5,6]. However, the downstream signaling pathways of Nods are not fully understood.

Recent evidence suggests that phosphatidylinositol 3-kinase (PI3K)/Akt pathway modulates Toll-like receptor signaling pathways. PI3K is a heterodimeric protein consisting of p85 regulatory subunit and p110 catalytic subunit. Activation of PI3K occurs through phosphorylation of tyrosine residues in the Src homology 2 domain of p85, which allows the association of PI3K to the plasma membrane and increases its catalytic activity through allosteric modifications [7,8]. Activated PI3K catalyzes the phosphorylation of membrane inositol lipids and the accumulation of phosphatidylinositol 3,4,5-trisphosphate and its phospholipid phosphatase product phosphatidylinositol 3,4-bisphosphate in the membrane, which recruits the lipid kinases phosphatidylinositol-dependent kinase 1 (PDK1) and Akt/protein kinase B [9–11]. After the membrane recruitment, Akt is fully activated by dual phosphorylation of Thr308 and Ser473 by PDK1 and mTOR/richtor complex (mTORC2), respectively [12–14]. It has been shown that wortmannin, a specific inhibitor of PI3K, enhanced LPS (TLR4 agonist)-induced nitric-oxide synthase in murine peritoneal macrophages [15]. Activation of PI3K/Akt in human monocytes limited LPS induction of TNFα and tissue factor expression to ensure transient expression of those potent inflammatory mediators [16]. Moreover, inhibition of PI3K enhanced TRIF-dependent NF-κB activation and IFN-β synthesis downstream of TLR3 and TLR4 [17]. PI3K was also found to negatively regulate flagellin-induced proinflammatory inducible NO synthase, interleukin (IL)-6 and IL-8 expression mediated by TLR5 [18]. In contrast to those negative regulations, PI3K has also been reported to play a critical role in activating cytokine expression mediated by TLR2 and TLR9 [19,20]. TLR2 stimulation by *Staphylococcus aureus* caused the activation of Rac1 and PI3K pathways, which targets nuclear p65 transcriptional activity, independently of IκBα degradation [19]. CpG DNA (TLR9 agonist) was shown to inhibit dendritic cell apoptosis by up-regulating cellular inhibitor of apoptosis proteins through the PI3K pathway to maintain immune response [20]. However, it has not been reported whether PI3K/Akt pathway is involved in Nod2 activation and how PI3K/Akt might regulate Nod2 signaling pathway.

Here, we show that Nod2 activation leads to activation of PI3K/Akt pathway and that PI3K/Akt pathway negatively regulates NF-κB activation and IL-8 expression induced by Nod2 activation.

2. Materials and methods

2.1. Reagents

MurNAc-L-Ala-D-isoGln was purchased from Bachem (King of Prussia, PA). Wortmannin and LY 294002 were purchased from Calbiochem (San Diego, CA). Sodium salts of saturated fatty acids and unsaturated fatty acids were purchased from Nu-chek (Eslyan, MN). They were dissolved in endotoxin-free water. Polyclonal NF-κB p65 and phospho-Ser536 p65, Akt, phospho-Thr308 Akt and phospho-Ser473 Akt antibody were purchased from Cell Signaling Technology (Danvers, MA). Phospho-Ser529 p65 antibody was from Rockland Inc. (Gilbertsville, PA). IκBα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin antibody was from Sigma (St. Louis, MO). All other reagents were purchased from Sigma unless otherwise indicated.

2.2. Cell culture

HEK293T (human embryonic kidney epithelial cells) was provided by Sam Lee (Beth Israel Hospital, MA). HEK293T cells stably transfected with Nod2, 293T-Nod2, were previously described [21]. Both of these cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Blastcidin (selection marker for Nod2 expression plasmid) was added for 293T-Nod2 cells. Human colonic epithelial cell lines HCT116, purchased from ATCC, were cultured in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

2.3. Plasmids

(2x)-NF-κB-dependent luciferase reporter construct (NF-κB-Luc), which contains two copies of NF-κB consensus-binding site, was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). Human IL-8 (−173 bp)-Luc was from Marie Annick Buendia (Institute Pasteur, France). pRSV-β-galatosidase reporter plasmid was from Jongdae Lee (University of California, San Diego, CA). An activated form of PI3K catalytic subunit, p110 (fernestylated form, p110(CA)) was from Marty Mayo (University of Virginia). A dominant-negative mutant of p85 (pSG5-p85ΔiSH2) and a dominant-negative mutant of Akt (SRα-Akt(T308A/S473A)) were obtained from Bing-Hua Jiang (West Virginia University, VA). A constitutively active form of Akt (myristoylated form, Akt(CA)) and wild-type Akt were provided by Michael Weber (University of Virginia Health Sciences Center, VA). p65-Gal4 and Gal4-Luc were from Nywana Sizemore (Cleveland Clinic Foundation, OH). All DNA constructs were prepared in large scale using the EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

2.4. Transfections and luciferase assays

For transient transfections and luciferase assays, 293T-Nod2 stable cells or HCT116 cells were seeded 4–5 × 10^4 cells in 24 well plates, and were co-transfected with 2xNF-κB-Luc or IL-8 (−173 bp)-Luc and pRSV-β-galactosidase plasmid using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Various expression plasmids for dominant negative or constitutive active forms of signaling components or their corresponding empty vector plasmids were co-transfected as indicated in the figures legends. The

total amount of plasmids was equalized by supplementing with the corresponding empty vector to eliminate the experimental errors from transfection itself. Luciferase and β-galactosidase enzyme activities were determined using Luciferase Assay System and β-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity to correct for the transfection efficiency.

2.5. *NF-κB-binding assay*

Activation of NF-κB was determined by TransAM NF-κB kit (Active Motif, Carlsbad, CA) according to the manufacture's instruction. Briefly, nuclear extracts of treated cells were incubated in 96-well plates coated with an oligonucleotide containing the NF-κB consensus sites for 1 h at room temperature. After three washes, NF-κB p65 or p50 antibody was added for 1 h followed by horseradish peroxidase-conjugated secondary antibody. NF-κB activation was determined by colorimetric reaction developed, stopped and measured at 450 nm with a reference at 650 nm.

2.6. *Immunoblotting*

The experiments were performed essentially as described previously [21,22]. The whole cell lysate were subjected to 8–

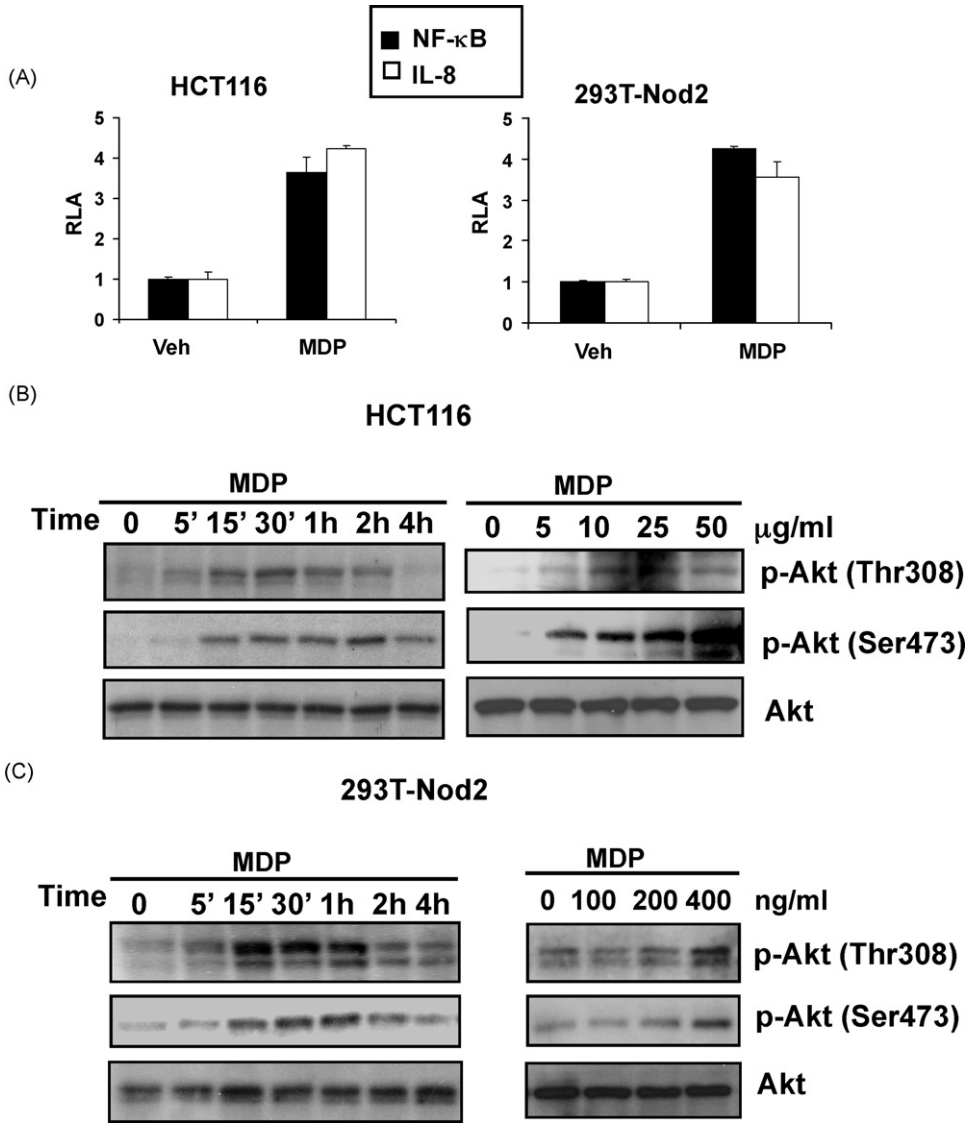

Fig. 1 – MDP dose dependently activates PI3K/Akt pathway in human colonic epithelial HCT116 cells and 293T stably transfected with Nod2. (A) MDP induces NF-κB and IL-8 transactivation in HCT116 and 293T stably transfected with Nod2. HCT16 (left panel) or 293T-Nod2 (right panel) were transfected with 2xNF-κB-Luc or IL-8-Luc reporter and β-galactosidase control plasmid and were stimulated with MDP (25 μg/ml for HCT116, 200 ng/ml for 293T-Nod2) for 15 h. The reporter gene assays were performed. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. (B and C) HCT116 (B) or 293T-Nod2 (C) were seeded and treated with MDP (25 μg/ml in B, 200 ng/ml in C) for indicated times or for increasing doses as indicated. The whole cell lysate were prepared for immunoblotting analysis for phospho-Thr308, phospho-Ser473 Akt and Akt.

10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked in 20 mM Tris–HCl, 137 mM NaCl, and 0.1% (v/v) Tween 20 (pH 7.4) containing 5% non-fat milk. The membrane was immunoblotted with primary antibodies for 1–24 h, followed by secondary antibody coupled to horseradish peroxidase (GE Healthcare, Piscataway, NJ) for 1 h. The membrane was exposed on an X-ray film using ECL Western blot detection reagents (GE Healthcare). To reprobe with different antibodies, the membrane was stripped in stripping buffer containing 62.5 mM Tris–HCl, 2% SDS and 100 mM 2-mercaptoethanol at 53 °C for 30–40 min.

2.7. Measurement of IL-8

The supernatants of HCT116 cells that were transient transfected for reporter gene assays and stimulated with different stimulus were collected and level of IL-8 were determined by enzyme-linked immunosorbent assay (ELISA) kits (OptEIA ELISA kits, BD Pharmingen, San Diego, CA).

2.8. Statistical analysis

Luciferase activity from reporter gene assays was normalized by β-galactosidase activity to yield relative luciferase activity (RLA). Data were shown as mean ± S.D. from three replicate experiments. The data were analyzed by *t*-test (two tailed) or one-way analysis of variance (ANOVA) using SigmaStat (Systat Software, San Jose, CA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Nod2 agonist muramyldipeptide activates PI3K/Akt pathway in human colonic epithelial HCT116 cells and 293T cells stably transfected with Nod2

It has been shown that the minimal bacterial PGN recognized by Nod2 to induce NF-κB activation is MurNAc-L-Ala-D-isoGln

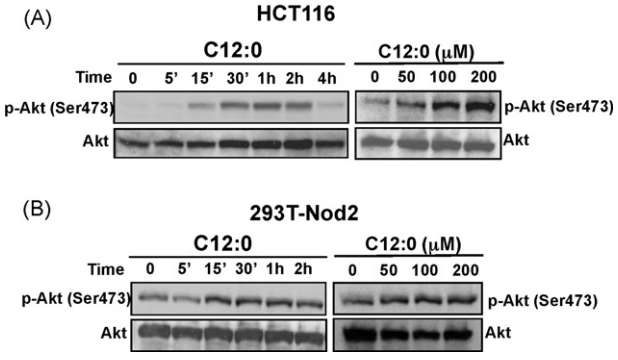

Fig. 2 – Saturated fatty acid lauric acid (C12:0) activates PI3K/Akt pathway in HCT116 and 293T stably transfected with Nod2. (A and B) HCT116 (A) or 293T-Nod2 (B) were seeded and treated with lauric acid (C12:0, 100 μM) for indicated times or increasing doses (50, 100 and 200 μM) for 30 min. The whole cell lysate were prepared for immunoblotting analysis for phospho-Ser473 Akt and Akt.

in 293T cells ectopically transfected with Nod2 expression plasmid [23,24]. We determined whether MDP can activate Nod2 in human colonic epithelial HCT116 cells, which express endogenous Nod2 [25], and 293T cells stably transfected with Nod2 (293T-Nod2). Indeed, MDP induced NF-κB and the target gene IL-8 transactivation in HCT116 and 293T-Nod2 cells (Fig. 1A). The higher MDP concentration was used in HCT116 compared with 293T-Nod2 stable cells, reflecting the fact the Nod2 expression is lower in HCT116 cells than that in 293T-Nod2 cells (data not shown). Next, we analyzed the ability of MDP to activate PI3K/Akt. MDP induced the activation of PI3K/Akt pathway in a time and dose-dependent manner as revealed by the phosphorylation of Akt on Thr308 and Ser473, the well-known downstream targets of PI3K/Akt pathway, in both HCT116 and 293T-Nod2 cells (Fig. 1B and C). These data suggest that activation of PI3K/Akt pathway is associated with Nod2 activation by MDP.

3.2. Saturated fatty acid (C12:0) activates PI3K/Akt pathway in HCT116 and 293T cells stably transfected with Nod2

We have previously shown that non-microbial molecule, saturated fatty acid lauric acid (C12:0) also activated Nod2 signaling pathway and induced NF-κB and IL-8 transactivation in HCT116 cells, which express endogenous Nod1 and Nod2, not detectable amount of TLR2 and TLR4, and 293T transfected with Nod1 or Nod2 [21]. These results suggest that Nod2 can also be activated by non-microbial molecule. Here, we determined whether lauric acid, as a non-microbial agonist for Nod2, activates PI3K/Akt pathway. Indeed, lauric acid induced Akt phosphorylation in similar time and dose-dependent manners in both HCT116 and 293T-Nod2 cells (Fig. 2A and B), demonstrating that PI3K/Akt pathway is activated by Nod2 agonists of both microbial and non-microbial origins.

3.3. The Inhibition of PI3K/Akt pathway leads to enhanced NF-κB activation and IL-8 expression induced by MDP in HCT116 and 293T stably transfected with Nod2

Next, we determined the functional consequences of PI3K/Akt activation on MDP-induced Nod2 activation by using PI3K pharmacological inhibitor, wortmannin, and the dominant negative or constitutively active forms of signaling components of PI3K/Akt pathway. Inhibition of PI3K by wortmannin resulted in enhanced NF-κB and IL-8 transactivation induced by MDP in both HCT116 and 293T-Nod2 cells, as revealed by NF-κB and IL-8 reporter gene assays (Fig. 3A and D). Wortmannin was also shown to enhance MDP-induced binding of κB consensus site by NF-κB p65 and p50, which is known to form the most common dimers found in the NF-κB signaling pathway, as revealed by NF-κB-binding assay (Fig. 3C and E). Wortmannin starting at 100 nM significantly enhanced IL-8 protein secretion in HCT116 cells ($P < 0.05$ for 100 nM, $P < 0.001$ for >100 nM) (Fig. 3B). Moreover, to confirm the results with PI3K inhibitor, we chose to use the specific dominant negative and constitutive active forms of the components of PI3K/Akt pathway. The inhibition of PI3K/Akt pathway by dominant-negative form of p85 (p85(DN)) or Akt (Akt(DN)) significantly enhanced, but constitutively active

form of p110 (p110(CA) inhibited, NF-κB and IL-8 transactivation induced by MDP and C12 (Fig. 4A–C). More robust effects of those mutant forms were seen with 293T-Nod2 stable cells than with HCT116, presumably due to the much higher transfection efficiency with 293T cells than with HCT116 cells (85–90% transfection efficiency with 293T cells vs. 20–30% transfection efficiency with HCT116 using SuperFect) (data not shown). Together, these results demonstrate that PI3K/Akt pathway negatively regulate Nod2 activation.

3.4. *The effects of PI3K pharmacological inhibitors on MDP-induced IκB degradation*

Since the activation of NF-κB is known to be regulated by at least two different mechanisms: the phosphorylation and consequent degradation of the inhibitor protein IκB and the phosphorylation of p65, a subunit of NF-κB, in a cooperative manner [26–28], we determined the roles of PI3K/Akt on both of these pathways downstream of Nod2 activation. First, we determined whether IκBα degradation was affected by PI3K/Akt pathway. While LY294002 did not significantly affect IκBα degradation, wortmannin enhanced IκBα degradation (Fig. 5A and B). Similar results were obtained from 293T-Nod2 cells (data not shown).

3.5. *The inhibition of PI3K/Akt pathway by the pharmacological inhibitors and by dominant-negative form of p85 or Akt enhance, but constitutively active form of p110 or Akt inhibit, the transactivation potential of p65 induced by MDP*

Next, we analyzed the effects of PI3K/Akt on the transactivation potential of p65 downstream of Nod2 activation by using p65-Gal4 (p65 fused with the activation domain of the

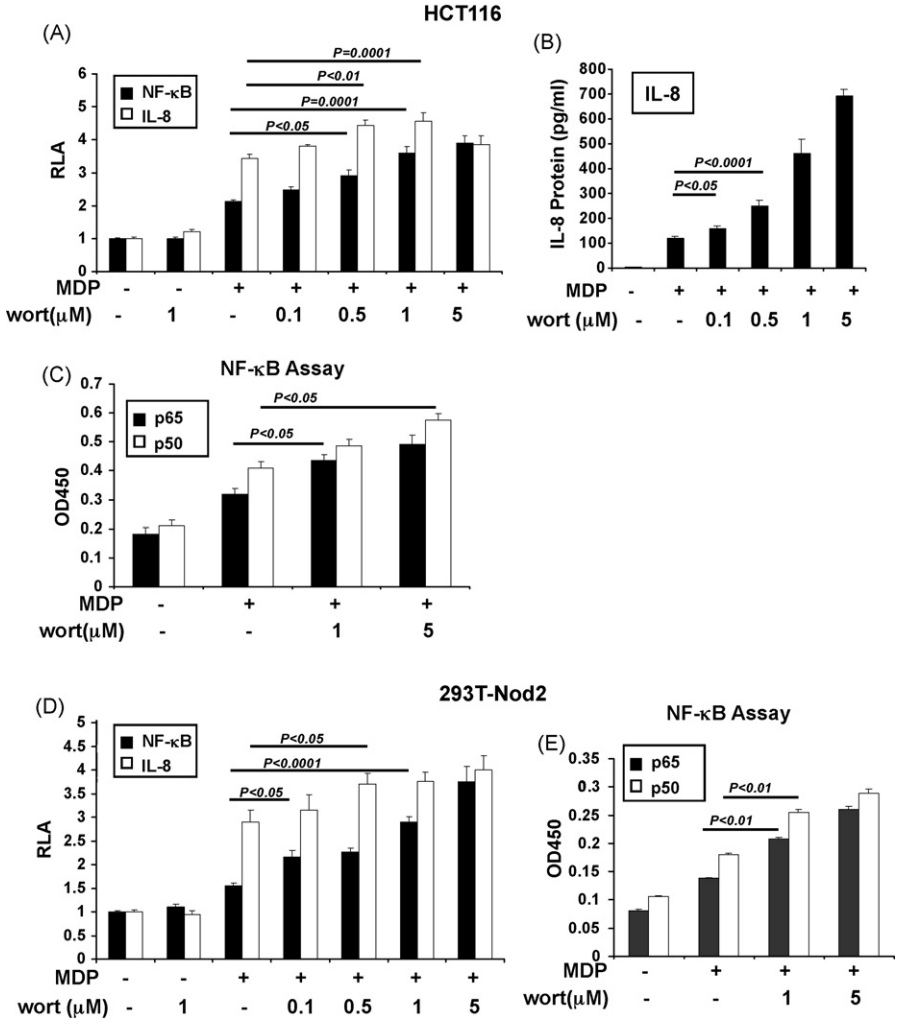

Fig. 3 – The effects of wortmannin on NF-κB activation and IL-8 expression induced by MDP in HCT116 and 293T stably transfected with Nod2. (A and D) HCT116 (A) or 293T-Nod2 stable cells (D) were transfected with 2xNF-κB-Luc or IL-8-Luc reporter and β-galactosidase and were pretreated with wortmannin (0.1, 0.5, 1 and 5 μM) or DMSO for 1 h and further co-treated with MDP for 15 h. The reporter assays were performed. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. (B) IL-8 protein secretion from HCT116 cells from (A) was analyzed by IL-8 ELISA. (C and E) HCT116 (C) and 293T-Nod2 (E) cells were seeded and pretreated with wortmanin (1 and 5 μM) for 1 h and further co-treated with MDP for 1 h. The nuclear extracts were prepared and subjected for NF-κB p65 and p50-binding assays, as described in Section 2.

transcription factor Gal4) and Gal4-Luc (a Gal4 responsive element linked to luciferase reporter). Both LY294002 and wortmannin dose dependently enhanced p65 transactivation induced by Nod2 activation (Fig. 6A). Moreover, p65 transactivation activity was also significantly enhanced by the transfection of dominant-negative form of p85 (p85(DN) or Akt (Akt(DN), but was significantly attenuated by the transfection of constitutively active form of p110 (p110(CA)) or Akt (Akt(CA)) (Fig. 6B). These data suggest that PI3K/Akt pathway negatively regulate MDP-induced NF-κB activation by inhibiting MDP-induced p65 transactivation activity.

3.6. *The inhibition of PI3K/Akt pathway attenuates Akt activation and enhances phosphorylation of NF-κB p65 on Ser529 and Ser536, the residues involved in transcriptional potential of p65*

To further determine the effects of PI3K/Akt on p65 transactivation potential downstream of Nod2 activation, we analyzed the phosphorylation of p65 on Ser529 and Ser536, the residues that were reported to be implicated in p65 transactivation potential [26,27,29]. As expected, inhibition of PI3K by LY294002 or wortmannin decreased the downstream Akt activation, as revealed by decreased phosphorylation of Akt on Ser473 and Thr308. MDP induced time-dependent phosphorylation of p65 on Ser529 and Ser536. LY294002 and wortmannin further enhanced phosphorylation of p65 on those residues in HCT116 cells (Fig. 7A and B). Similar results were seen in 293T-Nod2 cells (data not shown). Moreover, inhibition of PI3K/Akt pathway by over-expressing Akt(DN) was also shown to enhance MDP-induced phosphorylation of p65 on Ser529 and Ser536 (Fig. 7C). These results suggest that PI3K/Akt pathway negatively regulate MDP-induced NF-κB activation by inhibiting p65 transactivation potential.

3.7. *The PI3K pharmacological inhibitors abrogate GSK-3β inactivation induced by MDP*

To gain insights of how PI3K/Akt pathway might regulate NF-κB activation downstream of Nod2 activation, we focused on

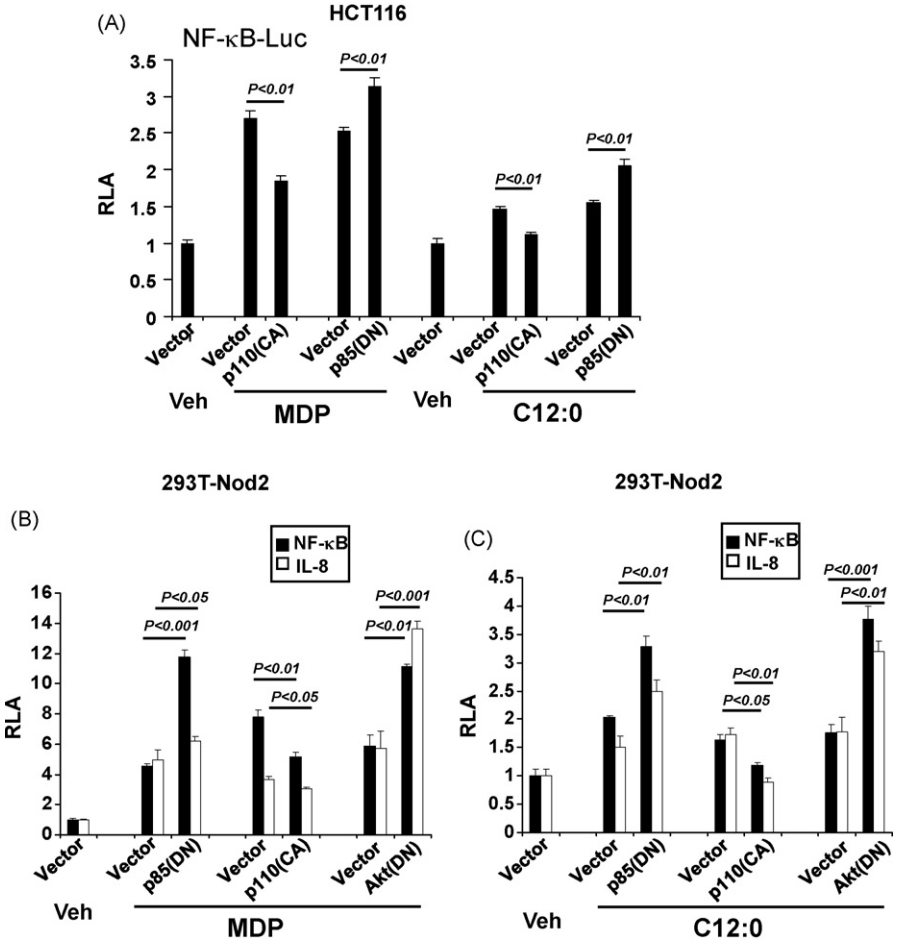

Fig. 4 – The inhibition of PI3K/Akt by dominant-negative form of p85 or Akt enhances, but constitutive active form of p110 or Akt inhibits, NF-κB and IL-8 transactivation induced by MDP and C12:0 in HCT116 and 293T stably transfected with Nod2. (A) HCT116 were transfected with 2xNF-κB-Luc and β-galactosidase together with p85(DN), p110(CA), or their corresponding vectors for 24 h. The cells were treated with MDP or lauric acid for 15 h. (B and C) 293T-Nod2 were transfected with 2xNF-κB-Luc or IL-8-Luc reporter and β-galactosidase together with p85(DN), p110(CA), Akt(DN) or their corresponding empty vector for 24 h. The cells were treated with MDP or lauric acid for 15 h. The reporter gene assays were performed. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity.

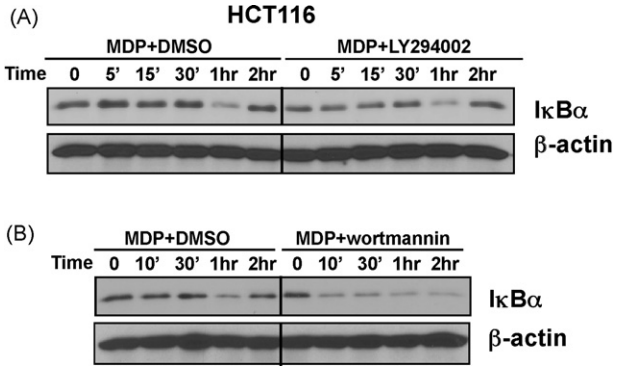

Fig. 5 – The effects of PI3K inhibition by LY294002 and wortmannin on IκB degradation induced by MDP in HCT116 cells. (A and B) HCT116 were seeded and pretreated with LY294002 (10 μM) (A), wortmannin (5 μM) (B) or DMSO for 1 h and co-treated with MDP for indicated times. The whole cell lysate were prepared for immunoblotting analysis for IκBα and β-actin.

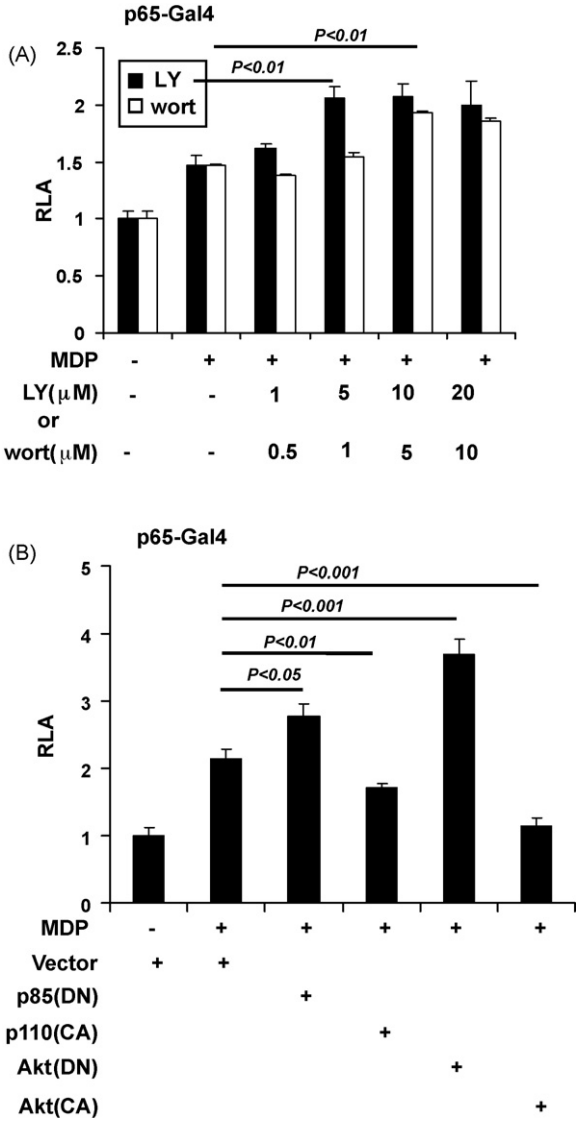

Fig. 6 – The inhibition of PI3K/Akt by inhibitors and by dominant-negative form of p85 or Akt enhance, but constitutive active form of p110 or Akt inhibit, the transactivation potential of p65 induced by MDP in 293T stably transfected with Nod2. (A) 293T-Nod2 cells were transfected with p65-Gal4 and Gal4-Luc together with β-galactosidase for 24 h. The cells were pretreated with increasing doses of LY294002 (1, 5, 10 and 20 μM), wortmannin (0.5, 1, 5 and 10 μM) or DMSO for 1 h and co-treated with MDP for 15 h. (B) 293T-Nod2 cells were transfected with p65-Gal4, Gal4-Luc, β-galactosidase and p85(DN), p110(CA), Akt(DN), Akt(CA) or their vectors for 24 h. The cells were treated with MDP for 15 h. The reporter gene assays were performed. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity.

GSK-3β, one of the Akt downstream targets. It has been shown that GSK-3β was inactivated by Akt through phosphorylation on Ser9 [30]. The phenotype of $GSK^{-/-}$ embryos is similar to that of $RelA^{-/-}$ embryos, suggesting that GSK-3β may regulate the transactivation activity of p65 [31]. Moreover, LPS-induced p65 transactivation activity was shown to be inhibited when GSK-3β was inactivated by LiCl, a known specific GSK-3β inhibitor. Pharmacological inhibitor of PI3K, LY294002, abrogated this GSK-3β inactivation downstream of LPS, suggesting that PI3K/Akt may negatively modulate GSK-3β thereby inactivating p65 transactivation downstream of LPS [16]. Therefore, we determined whether GSK-3β plays any role in the negative regulation of NF-κB by PI3K/Akt downstream of Nod2 activation. LiCl inhibited NF-κB and IL-8 activation induced by MDP in HCT116 cells (Fig. 8A). Moreover, LiCl also inhibited NF-κB p65 transactivation induced by MDP (Fig. 8B), suggesting that GSK-3β activity is required for NF-κB and IL-8 activation downstream of Nod2 activation. Moreover, we confirmed that GSK-3β phosphorylation on Ser9 was associated with GSK-3β inactivation by LiCl in HCT116 cells, as shown in Fig. 8C. We further studied the role of PI3K/Akt on GSK-3β activation downstream of Nod2 activation. MDP induced phosphorylation (inactivation) of GSK-3β on Ser9 in a time-dependent manner (Fig. 8D and E, left panels), which correlated with the kinetics of Akt activation by MDP (Fig. 1). Both LY294002 and wortmannin abrogated MDP-induced phosphorylation of GSK-3β, rendering the kinase in its active state (Fig. 8D and E, right panels). These results demonstrate that GSK-3β is inactivated in a PI3K/Akt-dependent manner upon Nod2 activation, suggesting the PI3K/Akt pathway may negatively regulate MDP-induced NF-κB through inactivation of GSK-3β.

4. Discussion

Our study demonstrates that PI3K/Akt pathway is activated by Nod2 activation; inhibition of PI3K/Akt leads to enhancement, but constitutive activation of the pathway leads to attenuation, of NF-κB activation and target gene IL-8 expression downstream of Nod2 activation. Our results suggest that PI3K/Akt pathway negatively regulate Nod2 activation and

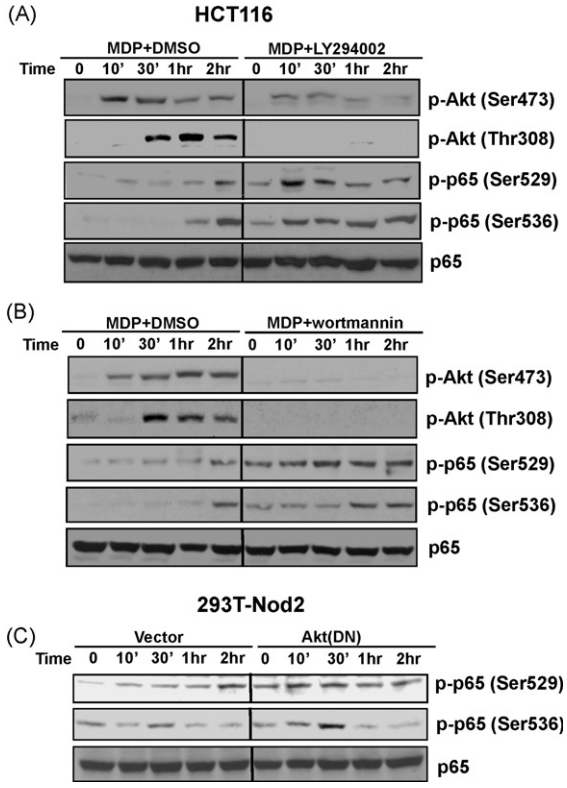

Fig. 7 – The inhibition of PI3K/Akt pathway attenuates Akt activation and enhances phosphorylation of NF-κB p65 on Ser529 and Ser536, the residues involved in transcriptional potential of p65. (A and B) HCT116 cells were seeded and pretreated with LY294002 (10 μM) (A), wortmannin (5 μM) (B) or DMSO for 1 h, further co-treated with MDP for indicated times. (C) 293T-Nod2 cells were transfected with Akt(DN) or the vector for 48 h and treated with MDP for indicated times. The whole cell lysate were prepared for immunoblotting analysis for phospho-Ser473 Akt, phospho-Thr308 Akt, phospho-Ser529 p65, phospho-Ser536 p65 and p65, as indicated.

may be involved in the resolution of inflammatory response induced by Nod2 activation. Recent reports have provided evidence that PI3K are a part of an intracellular control mechanism regulating the initial phases of innate immune response against diverse microbial pathogens. It has been shown that negatively modulation of the PI3K signaling pathway can reduce the morbidity and mortality associated with sepsis, inflammation, and ischemia/reperfusion injury *in vivo* [32–34]. The roles of PI3K/Akt pathway in limiting the inflammatory responses induced by TLRs have been demonstrated [16–18]. Here, we show that PI3K/Akt pathway also negatively regulates inflammatory responses induced by Nod2 activation. These results further suggest that PI3K/Akt pathway may be a common mechanism to resolve innate immune responses.

Wortmannin and LY294002, the pharmacological inhibitors of PI3K, have been widely used to examine the roles of PI3K in various cellular responses. In our study, the effects of wortmannin were seen from 100 nM and the effects of LY294002 were seen from 10 μM, both of which are typical concentrations used for those two inhibitors for inhibiting PI3K activity [35]. The effects of inhibitors were further confirmed by using specific dominant negative or constitutive active forms of components of PI3K/Akt pathway. Taken together, these data clearly demonstrate that the PI3K/Akt pathway negatively regulate NF-κB pathway downstream of Nod2 activation.

The roles of PI3K/Akt pathway in NF-κB-dependent gene expression are controversial. The PI3K/Akt pathway has been shown to act both positively and negatively on NF-κB-dependent gene expression downstream of TLR activation [16–20,22]. These differences may be due to the use of different cell types and/or different stimulations. For example, in human monocytic cells, TLR2-dependent PI3K/Akt pathway was shown to positively regulate the transactivation potential of p65. However, PI3K/Akt negatively regulated p65 transactivation induced by TLR4 activation [16,19]. These data reflects the specificity of the effects of PI3K/Akt on different signaling pathways.

It remains to be determined how PI3K/Akt negatively regulates NF-κB activation downstream of Nod2 activation. We show that PI3K inhibition by wortmannin enhance both IκB degradation and p65 transactivation potential. Moreover, we show that the increased p65 transactivation in the presence of PI3K inhibitors correlate with the activation of glycogen synthase kinase (GSK)-3β in the presence of those inhibitors, suggesting that PI3K/Akt may negatively regulate NF-κB p65 transactivation via the inactivation of GSK-3β, similar to the negative regulation of PI3K/Akt on LPS signaling pathway [16]. Moreover, PI3K/Akt pathway was shown to negatively modulate MAPK pathway activated by TLR4 or TLR5 agonist, leading to diminished pro-inflammatory gene expression [16,18]. It remains to be determined whether MAPK pathway, in addition to NF-κB pathway, is also modulated by PI3K/Akt downstream of Nod2 activation.

It is well recognized that despite the beneficial effects of inflammation in host defense against infection and local injuries, the resolution of the inflammation is also essential to prevent chronic inflammation, maintaining the balance between health and disease. When the homeostatic regulatory mechanisms are perturbed, the risks of developing chronic inflammatory diseases are increased. In Crohn's disease (CD), a chronic relapsing inflammatory disease of the bowel, three major mutations in Nod2 gene (R702W, G908R, L1007fsincC) have been found to be associated with increased susceptibility [36,37]. These CD associated Nod2 variants are deficient in their ability to sense and respond to PGN and/or synthetic MDP despite the fact that there is elevated inflammation in those patients [23,24,38,39]. It needs to be determined whether the resolution mechanism induced by Nod2 activation plays any part in the development of Crohn's disease and whether enhancement of the resolution mechanism may be of therapeutic value in the treatment of the disease.

In conclusion, our results demonstrate that the PI3K/Akt pathway negatively regulates NF-κB activation downstream of Nod2 activation. Our results suggest that PI3K/Akt may

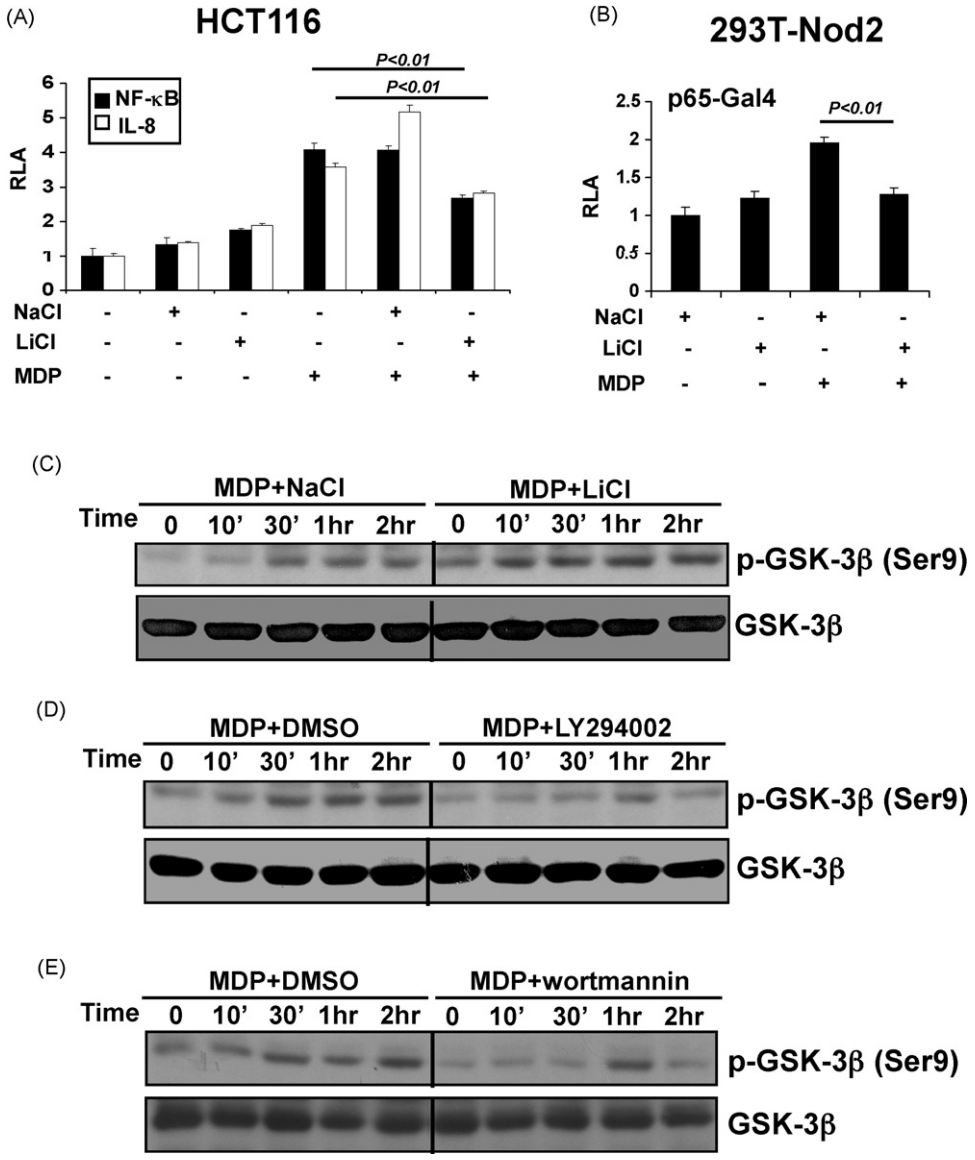

Fig. 8 – MDP-induced NF-κB activation is mediated through GSK-3β and the inhibition of PI3K by LY294002 or wortmannin abrogates the inhibitory phosphorylation of GSK-3β induced by MDP. (A and B) HCT116 cells (A) were transfected with NF-κB or IL-8-Luc. 293T-Nod2 stable cells (B) were transfected with p65-Gal4 and Gal4-Luc. The cells were pretreated with LiCl (50 mM), NaCl (50 mM) or vehicle for 1 h, and further co-treated with MDP for 15 h. The reporter assays were performed. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. (C–E) HCT116 cells were seeded and pretreated with NaCl or LiCl for 1 h and further co-treated with MDP for indicated times (C), or pretreated with LY294002 (10 μM) (D), wortmannin (5 μM) (E) or DMSO for 1 h, further co-treated with MDP for indicated times. The whole cell lysate were prepared for immunoblotting analysis for phospho-Ser9 GSK-3β and GSK-3β.

function as a mechanism for the resolution of the innate immune responses downstream of Nod2 activation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.12.014.

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