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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2-mediated ERK Signaling Inhibits Gram-negative Bacteria-induced RelA Phosphorylation and Interleukin-6 Gene Expression in Intestinal Epithelial Cells through Modulation of Protein Phosphatase 2A Activity*

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We have previously shown that non-pathogenic Gram-negative *Bacteroides vulgatus* induces transient RelA phosphorylation (Ser-536), NF-κB activity, and proinflammatory gene expression in native and intestinal epithelial cell (IEC) lines. We now demonstrate that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) but not prostaglandin E_2 inhibits lipopolysaccharide (LPS) (*B. vulgatus*)/LPS (*Escherichia coli*)-induced RelA phosphorylation and interleukin-6 gene expression in the colonic epithelial cell line CMT-93. This inhibitory effect of 15d-PGJ_2 was mediated independently of LPS-induced IκBα phosphorylation/degradation and RelA nuclear translocation as well as RelA DNA binding activity. Interestingly, although *B. vulgatus* induced nuclear expression of peroxisome proliferator-activated receptor γ (PPARγ) in native epithelium of monoassociated Fisher rats, PPARγ-specific knock-down in CMT-93 cells using small interference RNA failed to reverse the inhibitory effects of PPARγ agonist 15d-PGJ_2, suggesting PPARγ-independent mechanisms. In addition, 15d-PGJ_2 but not the synthetic high affinity PPARγ ligand rosiglitazone triggered ERK1/2 phosphorylation in IEC, and most importantly, MEK1 inhibitor PD98059 reversed the inhibitory effect of 15dPGJ_2 on LPS-induced RelA phosphorylation and interleukin-6 gene expression. Calyculin A, a specific phosphoserine/phosphothreonine phosphatase inhibitor increased the basal phosphorylation of RelA and reversed the inhibitory effect of 15d-PGJ_2 on LPS-induced RelA phosphorylation. We further demonstrated in co-immunoprecipitation experiments that 15d-PGJ_2 triggered protein phosphatase 2A activity, which directly dephosphorylated RelA in LPS-stimulated CMT-93 cells. We concluded that 15d-PGJ_2 may help to control NF-κB signaling and normal intestinal homeostasis to the enteric microflora by modulating RelA phosphorylation in IEC through altered protein phosphatase 2A activity.

The mucosal surfaces and cavities of the gastrointestinal tract in humans and animals are populated by a complex mixture of non-pathogenic microorganisms of more than 400 species having spatial differences in population size and relative species predominance along the digestive tract (1–4). The host has evolved various homeostatic mechanisms to acquire tolerance (hyporesponsiveness) to resident enteric microorganisms, whereas protective cell-mediated and humoral immune responses to enteropathogens are maintained. This complex homeostasis toward the normal enteric microflora is broken under conditions of chronic intestinal inflammation including the chronically relapsing, immune-mediated idiopathic disorders ulcerative colitis and Crohn's disease (5, 6). The selective effect of microbial factors in initiating and perpetuating chronic intestinal inflammation is extensively supported in comparative studies using germ-free and gnotobiotic rodent models for experimental colitis (7). For example, reconstitution studies with various non-pathogenic bacteria implicate *Bacteroides vulgatus* as particularly important to the induction of experimental colitis in monoassociated HLA-B27 transgenic rats (8, 9).

Increased NF-κB activity has been well documented in intestinal epithelial cell (IEC)[1] and lamina propria cells of inflammatory bowel disease patients with active disease (10–12), and accordingly, pharmacological NF-κB blockade may become potentially important in the treatment of chronic intestinal inflammation (13, 14). Indeed, local administration of antisense RelA oligonucleotides abrogated clinical and histological signs of trinitrobenzene sulfonic acid-induced experimental colitis, suggesting a mechanistic role for sustained NF-κB activity in the pathogenesis of chronic mucosal inflammation (15). On the other hand, blocking NF-κB activity with pharmacological inhibitors during the resolution phase of carrageenan-induced acute inflammation is deleterious to the host (16), suggesting dual functions of activated NF-κB including protective and detrimental mechanisms during the course of inflammation.

The induction of the IκB/NF-κB system and NF-κB-dependent gene expression is a complex process that involves the participation of multiple adaptor proteins and kinases acting in a coordinate fashion to give specificity to the cell surface stimuli. We have previously shown that *B. vulgatus* and lipopoly-

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[1] The abbreviations used are: IEC, intestinal epithelial cell(s); NF-κB, nuclear factor κB; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPARγ, peroxisome proliferator-activated receptor γ; PP2A, protein phosphatase 2A; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IKK, IκB kinase complex; TZD, thiazolidinedione; IL-6, interleukin 6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interference RNA; ELISA, enzyme-linked immunosorbent assay.

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saccharide (LPS) signal through the TLR4 cascade to trigger IRAK1 degradation and IκBα phosphorylation/degradation as well as NF-κB DNA binding activity and NF-κB transcriptional activity in native and IEC lines (17). In addition to the activation of the IκB/NF-κB system and nuclear translocation of transcriptionally active RelA, the modification of NF-κB transcriptional activity by phosphorylation of RelA at various serine residues (Ser-276, Ser-529, Ser-536) has been shown to be an important regulatory element of this signaling pathway (18–21). Potential kinases involved in signal-induced RelA phosphorylation are the casein kinase II, Akt, and IKK. We showed that IKKβ and the phosphatidylinositol 3-kinase/Akt pathway participate in *B. vulgatus*-induced phosphorylation of serine 529 and/or 536 of the RelA transactivating domain 1 (TAD1) in IEC (17). Most importantly for the physiological relevance, we showed that monoassociation of wild type rats with *B. vulgatus* triggered transient nuclear localization of phosphorylated (Ser-536) and transcriptionally active NF-κB subunit RelA (p65) in the intestinal epithelium (17, 22). The absence of colitis and pathological immune responses in *B. vulgatus*-monoassociated wild type rats confirmed the non-pathogenic nature of this obligate anaerobic Gram-negative bacterial strain and suggests that the normal host developed mechanisms to control NF-κB activity in IEC (23).

Protein serine/threonine phosphatases including PP1, PP2A, PP2B, and PP2C are involved in the regulation of signaling pathways with a variety of protein kinases (24, 25). PP2A has been shown to form a complex with calcium/calmodulin-dependent protein kinase IV (26), casein kinase (27), p21-activated kinase-1 and -3, and p70 S6 kinase (28) as well as certain G-protein-coupled receptors (29, 30). Recently, PP2A was shown to interact with and directly dephosphorylate RelA, suggesting PP2A as an important regulator of NF-κB signaling (31, 32). The predominant heterotrimeric form of PP2A consists of the 36-kDa catalytic subunit (PP2Ac) and a 65-kDa regulatory subunit (PP2Aa or PR65). In addition, several regulatory subunits are associated with the core enzyme, conferring substrate specificity to its dephosphorylating activity (24, 25).

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the steroid receptor superfamily with various cellular functions including differentiation, apoptosis, lipid metabolism, and anti-inflammatory responses (33–35). Although PPARγ is expressed in multiple tissues, the highest levels are found in adipose tissue and colonic epithelium (36). Ligand-specific activation of the PPARγ transcription factor has been shown to inhibit pro-inflammatory gene expression and experimental colitis for synthetic anti-diabetic thiazolidinediones (TZDs) including rosiglitazone and troglizitazone as well as the endogenous prostaglandin D$_2$ metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) (37–42). It appears from several studies that TZD and 15d-PGJ$_2$, which is present *in vivo* during the resolution phase of acute inflammation (43), mediate their anti-inflammatory effects also through PPARγ-independent mechanisms affecting the NF-κB signaling pathway at the level of IKK activity and IκBα degradation as well as RelA DNA binding activity (37, 44, 45).

In this study we characterized the molecular mechanism for the inhibitory effect of 15d-PGJ$_2$ on RelA phosphorylation (Ser-536) and IL-6 gene expression in IEC. Consistent with the transient induction of phospho-RelA (Ser-536) in the intestinal epithelium of *B. vulgatus*-monoassociated Fisher rats, 15d-PGJ$_2$ but not the synthetic high affinity PPARγ ligand rosiglitazone inhibited LPS (*B. vulgatus*)/LPS (*Escherichia coli*)-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells. Although *B. vulgatus* triggered nuclear expression of PPARγ in native epithelium of monoassociated Fisher rats, PPARγ-specific knock-down in CMT-93 cells using small interference RNA failed to reverse the inhibitory effects of PPARγ agonist 15d-PGJ$_2$, suggesting PPARγ-independent mechanisms. Finally, we could demonstrate that 15d-PGJ$_2$ inhibits LPS-induced RelA phosphorylation and IL-6 gene expression in IEC through the induction of the ERK signaling cascade by modulating PP2A activity.

MATERIALS AND METHODS

Animals and Bacterial Monoassociation—Germ-free Fisher F344 rats were monoassociated at 10–12 weeks of age with *B. vulgatus* (a generous gift from Dr. A. B. Onderdonk, Harvard University, Cambridge, MA) and maintained in the Gnotobiotic Animal Core at the College of Veterinary Medicine, North Carolina State University (Raleigh, NC). Bacterial monoassociation and the absence of contamination by other bacterial species were confirmed by culturing samples from the large intestine at necropsy and culturing serial fecal samples. Animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC), North Carolina State University. Rats were killed 3, 7, 14, and 35 days after initial bacterial colonization. Germ-free mice were used as controls. Sections of the ileum, cecum, proximal, and distal colon were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin. Histology scoring was analyzed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion.

Immunohistochemistry and Isolation of Primary Rat Intestinal Epithelial Cells—*B. vulgatus*-monoassociated and germ-free rats were euthanized, and the cecum as well as colon were removed and placed in Dulbecco's modified Eagle's medium (Invitrogen) containing 5% fetal calf serum. Cecum and colon were cut longitudinally, washed three times in calcium/magnesium-free Hanks' balanced salt solution (Invitrogen), cut into pieces 0.5 cm long, and incubated at 37 °C in 40 ml of Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 1 mM dithiothreitol for 30 min in an orbital shaker. The supernatant was filtered and centrifuged for 5 min at 400 × *g*, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The remaining tissue was incubated in 30 ml of phosphate-buffered saline (1×) containing 1.5 mM EDTA for an additional 10 min. The supernatant was filtered and centrifuged for 5 min at 400 × *g*, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Finally, primary IEC were collected by centrifugation through a 25/40% discontinuous Percoll gradient at 600 × *g* for 30 min. Cell viability and purity was assessed by trypan blue exclusion and fluorescence-activated cell sorter analysis using mouse anti-CD3 monoclonal antibody (BD Biosciences Pharmingen, clone G4.18). Cells were >80% viable and >90% pure. Primary rat IEC from cecum and colon were combined and collected in sample buffer for subsequent Western blot analysis. Immunohistochemistry on paraffin-embedded tissue sections was performed using anti-PPARγ Ab (Cell Signaling, Beverly, MA) according to the protocol of the manufacturer, and sections were counterstained with hematoxylin.

Cell Culture and Bacterial Infection—The IEC line CMT-93 (passage 10–30) (ATCC CRL 223, American Type Culture Collection) was grown in a humidified 5% CO$_2$ atmosphere at 37 °C to confluency in 6-well tissue culture plates (Cell Star, Greiner bio-one, Frickenhausen, Germany) as previously described (17). *B. vulgatus* was anaerobically grown at 37 °C in brain-heart infusion broth. Bacteria were harvested by centrifugation (3000 × *g*, 15 min) supplemented with cysteine (0.05%), hemin (5 mg/liter), and resazurin at stationary growth phase. For LPS purification, *B. vulgatus* was killed by the addition of 1% phenol (Fluka, Heidelberg) and washed thoroughly with twice-distilled water. Endotoxin was extracted and purified by the method of Westphal *et al.* (26). Purity was checked by SDS-PAGE (46). Confluent epithelial cell monolayers were stimulated with *B. vulgatus* LPS (10 μg/ml) and *E. coli* LPS (10 μg/ml; from *E. coli* serotype O111:B4, Sigma) in the presence or absence of 20 μM 15d-PGJ$_2$ (BioMol, Plymouth Meeting, PA) and 20 μM rosiglitazone (BioMol). Where indicated, cells were treated with 20 μM MEK1 inhibitor PD98059 (Calbiochem) or 1–5 nM calyculin A (Cell Signaling).

RNA Isolation and Real-time Reverse Transcription-PCR—RNA from IEC was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNA was dissolved in 20 μl of water containing 0.1% diethyl pyrocarbonate. For reverse transcription, 1 μg of total RNA was added to 30 μl of reaction buffer containing 8 μl of 5× first-strand buffer, 4 μl of dithiothreitol (100 mM), and 6 μl of deoxyribonucleoside triphosphate mixture (300 μM) (all reagents from Invitrogen) and incubated for 5 min at 65 °C. After adding 10 μl of a

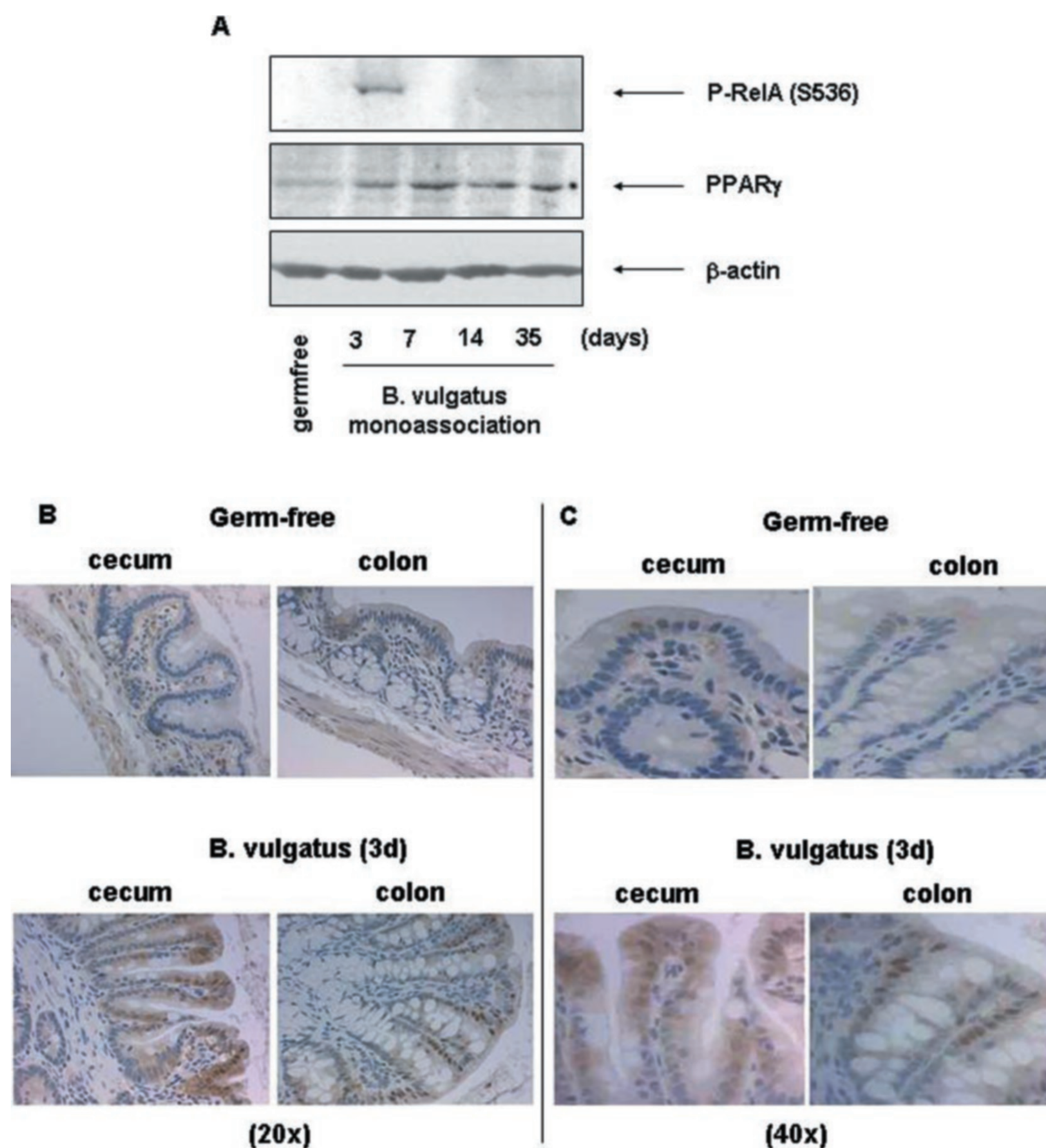

FIG. 1. **RelA phosphorylation and PPARγ expression in intestinal epithelium of *B. vulgatus*-monoassociated germ-free Fisher rats.** Germ-free Fisher rats (n = 2) were monoassociated with *B. vulgatus*. Rats were killed at days 3, 7, 14, and 35 after initial bacterial colonization. Native IEC were isolated from cecum and colon. Total protein was extracted, and 20 μg protein were subjected to SDS-PAGE followed by phospho-RelA, PPARγ, and β-actin immunoblotting using ECL technique (*A*). Sections of cecum and colon were fixed in 10% neutral-buffered formalin. Immunohistochemistry was performed on paraffin-embedded tissue sections using anti-PPARγ antibody and analyzed at 20× or 40× magnification (*B* and *C*). Sections were counterstained with hematoxylin.

solution containing 0.2 μg of random hexamers, 40 units of RNase Out, and 200 units of Moloney murine leukemia virus reverse transcriptase (all reagents from Invitrogen), the total mixture was incubated for an additional 60 min at 37 °C followed by a final 1-min heating step at 99 °C.

Real-time PCR was performed in glass capillaries using a Light Cycler™ system (Roche Diagnostics). Primer sequences and amplicon sizes are as follows: IL-6, 5′-acaacgatgatgcactt-3′ (forward) and 5′cttg-gtccttagccact-3′ (reverse) (334 bp); IκBα, 5′-gtgactttgggtgctg-3′ (forward) and 5′-gctgtatccgggtactt-3′ (reverse) (193 bp); PPARγ, 5′-tccgta-gaagccgtgc-3′ (forward) and 5′-ggatgtcctcgatggg-3′ (reverse) (364 bp); GAPDH, 5′-atcccagagctgaacg-3′ (forward) and 5′-gaagtcgcaggagaca-3′ (reverse) (198 bp). For real-time PCR, 1 μl of reverse-transcribed cDNA was added in a total volume of 10 μl of PCR reaction buffer containing 1× LC-FastStart DNA Master Mix (Roche Applied Science), $MgCl_2$ (4 μM), and forward and reverse primers (20 μM). The PCR program was one cycle of denaturation at 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. The amplified product was detected by the presence of a SYBR green fluorescent signal. Melting curve analysis and gel electrophoresis was used to document the amplicon specificity. Calibration curves were generated by measuring serial dilutions of stock cDNA to calculate the amplification efficiency (*E*). The crossing point (Cp) of the log-linear portion of the amplification curve was determined. The relative induction of gene mRNA expression was determined using the calculation $E^{\Delta Cp\ (\text{control samples} - \text{treated samples})}$ and normalized for the expression of GAPDH mRNA (47). Triplicate samples were measured in duplicate and blotted as -fold increase between treated and untreated control samples.

Western Blot Analysis—IEC were lysed in 1× Laemmli buffer, and 20–50 μg of protein was subjected to electrophoresis on 10% SDS-PAGE gels. Where indicated IEC cells were pretreated for 1 h with 20 μM proteasome inhibitor MG132 (BioMol). Anti-IκBα (C21, Santa Cruz Biotechnology), anti-NF-κB RelA (Santa Cruz), anti-phospho-IκBα (Ser-32), anti-phospho-RelA (Ser-536), anti-phospho-ERK1/2 (p44/p42) (Tyr-202/204), ERK1/2, anti-PPARγ (all antibodies from Cell Signaling, Beverly, MA), anti-PP2A (Abcam, Cambridge, UK), and anti-β-actin (ICN, Costa Mesa, CA) were used to detect immunoreactive phospho-IκBα, total IκBα, total RelA, phospho-RelA, phospho-ERK1/2, ERK1/2, PPARγ, PP2A, and β-actin, respectively, using an enhanced chemiluminescence light-detecting kit (Amersham Biosciences) as previously described (17).

Small Interference RNA and Transfection—Synthetic PPARγ-specific (accession number NM_011146) siRNA was designed and pur-

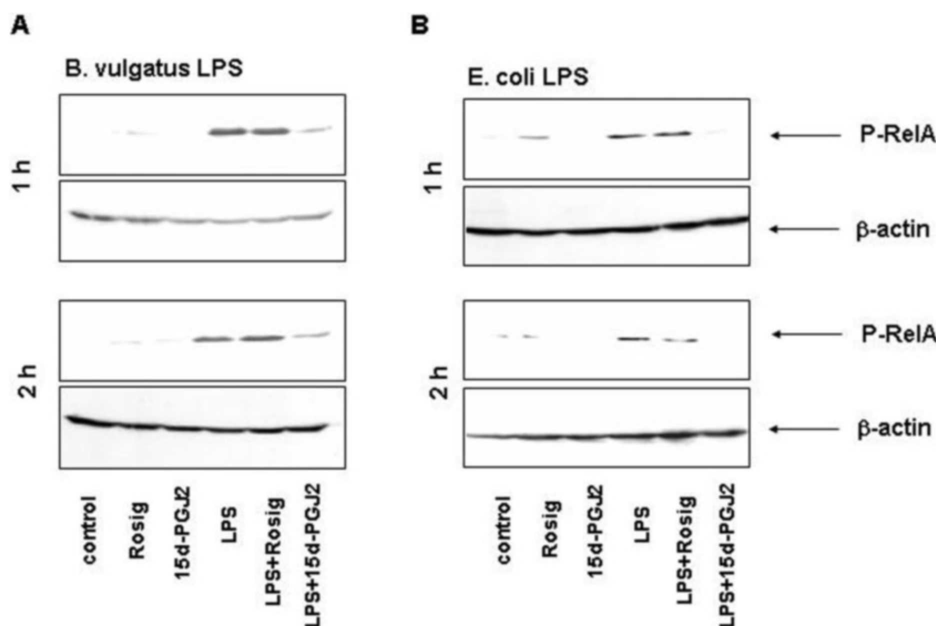

FIG. 2. **15d-PGJ$_2$ inhibits LPS-induced RelA phosphorylation in CMT-93 cells.** CMT-93 cells were stimulated with 10 μg/ml LPS from *B. vulgatus* (*A*) and *E. coli* (*B*) for 1 and 2 h in the presence of 20 μM 15d-PGJ$_2$ and 20 μM rosiglitazone (*Rosig*). Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-RelA and β-actin immunoblotting using ECL technique. The results are representative of three independent experiments.

chased from Qiagen (Hilden, Germany) according to the protocol of the manufacturer. The sequence was as follows: sense, 5′-AGACCCAG-CUCUACAACAG(TT)-3′; reverse 5′-CUGUUGUAGAGCUGGGUCU-(TT)-3′. The annealed double stranded ribooligonucleotides were dissolved in RNase-free buffer and stored at −20 °C in a concentration of 20 μM. Before performing the experiments, the siRNA was heat-treated for 1 min at 90 °C and then incubated for an additional 60 min at 37 °C. CMT-93 cells, which were grown to 80–90% confluency, were transfected with single-stranded siRNA (0.4, 0.8, or 1.6 μg of total siRNA) according to the protocol of the manufacturer using TransMessanger reagent (Qiagen). Finally, the transfected CMT-93 cells were cultured for an additional 48 h and then stimulated with LPS (10 μg/ml) for 12 h in the presence or absence of 15d-PGJ$_2$. Rhodamine-stained siRNA was used to visualize the cellular distribution in transfected CMT-93 epithelial monolayers using fluorescence microscopy.

Nuclear Extracts and NF-κB p65 Protein/DNA Binding Activity—CMT-93 cells were stimulated for various times (0–4 h) with *E. coli* LPS (10 μg/ml), and nuclear extracts were prepared according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Extracts (5 μg) were used to determine nuclear RelA binding activity to the κB-nucleotide consensus sequence 5′-GGGACTTTCC-3′ by using the TransAM ELISA-based NF-κB transcription factor assay (Active Motif). Protein/oligonucleotide binding activity was quantified by colorimetric analysis using a MultiScan spectrophotometer.

Co-immunoprecipitation and Phosphatase Activity Assay—CMT-93 cells were stimulated with either *E. coli* LPS (10 μg/ml) or 15d-PGJ$_2$ (20 μM). Cells were lysed in lysis buffer (50 mM Tris at pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, and 10% glycerol supplemented with protease inhibitors. Cell debris was removed by centrifugation at 12,000 × *g*, and the supernatant was precleared for 1 h with 20 μl of protein A/G-agarose (Santa, Cruz, Europe). Total protein concentration was normalized, and immunoprecipitation was carried out overnight at 4 °C using 5 μl of rabbit anti-phospho-RelA and goat anti-PP2A antibody (Abcam). Immune complexes were collected with 30 μl of protein A/G-agarose for 30 min, washed, and resuspended in reaction buffer (100 μl) containing 20 mM HEPES (pH 7.4), 10 mM MgCl$_2$, and 1 μM dithiothreitol. To assay the phosphatase activity, 30 μl of beads from LPS-stimulated cells were co-incubated with 50 μl of beads from 15d-PGJ$_2$-treated cells. Reactions were stopped after 1 and 2 h of incubation at 37 °C with the addition 2× Laemmli sample buffer. Western blot analysis for phospho-RelA and PP2A were carried out as described above.

ELISA Analysis—Protein concentrations were determined in spent culture supernatants of IEC cultures using an ELISA technique. IL-6 protein production was determined by mouse-specific ELISA assay kits according to the manufacturer's instructions (R&D Systems, Heidelberg, Germany).

Statistical Analysis—Data are expressed as the mean ± S.D. of triplicates. Statistical analysis was performed by the two-tailed Student's *t* test for paired data and considered significant if *p* values were <0.05 (*) or <0.01 (**).

RESULTS

B. vulgatus-monoassociated Fisher Rats Trigger Transient Phosphorylation of RelA and Persistent Expression of PPAR-γ in Native IEC—We investigated RelA phosphorylation and PPAR-γ expression in the intestinal epithelium of *B. vulgatus*-monoassociated and germ-free Fisher rats. The rats were killed after 3, 7, 14, and 35 days of initial bacterial colonization, and native IEC were isolated from cecal and colonic tissue. Phospho-RelA (Ser-536) and PPARγ protein expression were measured in isolated native IEC using Western blot analysis. As shown in Fig. 1, although *B. vulgatus* monoassociation of germ-free rats triggered transient phosphorylation of RelA at day 3 in native IEC, PPARγ protein expression was persistently induced 3–28 days after bacterial colonization. Nuclear localization of PPARγ in cecal and colonic epithelium was confirmed in intestinal tissue sections by performing immunohistochemical analysis (Fig. 1, *B* and *C*).

15d-PGJ$_2$ but Not Rosiglitazone Inhibits RelA Phosphorylation in CMT-93 Cells after Stimulation with B. vulgatus and E. coli LPS—We have previously shown that whole *B. vulgatus* cells and *E. coli*-derived LPS trigger RelA phosphorylation, NF-κB activity, and pro-inflammatory gene expression in IEC lines. Based on these results, we next asked the question whether PPARγ agonists inhibit RelA phosphorylation in CMT-93 cells. We used the endogenous prostaglandin D$_2$ metabolite 15d-PGJ$_2$ and the synthetic PPARγ ligand rosiglitazone to antagonize *B. vulgatus* LPS- and *E. coli* LPS-induced RelA phosphorylation in CMT-93. Fig. 2 shows that 15d-PGJ$_2$ inhibits RelA phosphorylation in CMT-93 cells after 1 and 2 h of stimulation with *B. vulgatus* LPS (Fig. 2*A*) as well as *E. coli* LPS (Fig. 2*B*). Of note, the synthetic high affinity PPARγ ligand rosiglitazone did not inhibit LPS-induced RelA phosphorylation. To further characterize the inhibitory mechanisms of 15d-PGJ$_2$ on LPS-induced NF-κB signaling, we used the commercially available *E. coli* LPS.

15d-PGJ$_2$ Failed to Inhibit LPS-induced IκBα Degradation, RelA Nuclear Translocation, and RelA DNA Binding Activity in IEC—We next investigated the effect of 15d-PGJ$_2$ and rosiglitazone on LPS-induced IκBα degradation, RelA nuclear translocation, and RelA DNA binding activity in CMT-93 cells. We stimulated the cells with LPS in the presence or absence of 15d-PGJ$_2$ and rosiglitazone for 1 and 2 h. Fig. 3*A* shows that

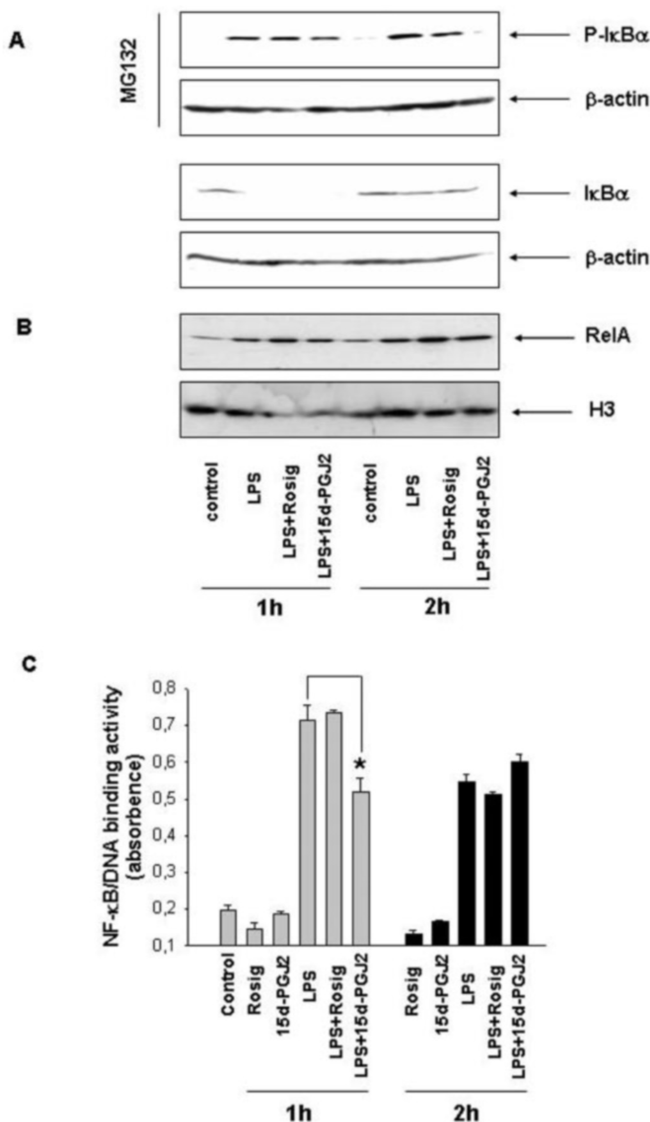

FIG. 3. **Effects of 15d-PGJ_2 on LPS-induced IκBα phosphorylation/degradation, RelA nuclear translocation, and RelA DNA binding activity.** CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* for 1 and 2 h in the presence or absence of 20 μM 15d-PGJ_2 and 20 μM rosiglitazone (*Rosig*). Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-IκBα, IκBα, and β-actin immunoblotting using an ECL technique (*A*). Nuclear extracts were isolated, and 20 μg of nuclear protein were subjected to SDS-PAGE followed by RelA and β-actin immunoblotting using ECL technique (*B*). Nuclear extracts were isolated, and RelA DNA binding activity was quantified using a TransAM ELISA-based NF-κB transcription factor assay. Protein/oligonucleotides binding activity was determined by colorimetric analysis using 5 μg of nuclear extracts (*C*). The results are representative three independent experiments.

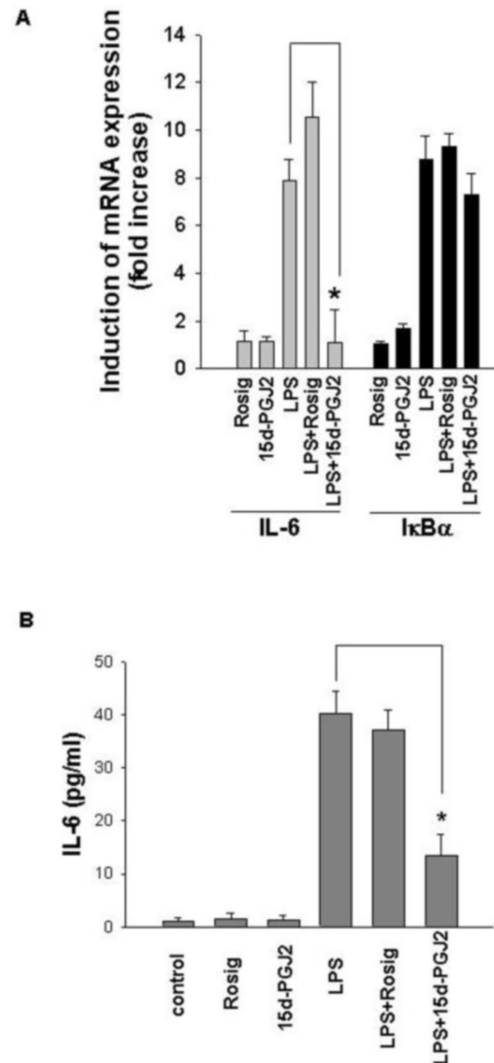

FIG. 4. **15d-PGJ_2 inhibits LPS-induced IL-6 mRNA but not IκBα gene expression.** CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* for 12 h in the presence or absence of 20 μM 15d-PGJ_2 and 20 μM rosiglitazone (*Rosig*). Total RNA was extracted and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6, IκBα, and GAPDH. The induction of IL-6 and IκBα mRNA expression was calculated relative to untreated controls (-fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH (*A*). CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* for 36 h in the presence or absence of 20 μM 15d-PGJ_2 and rosiglitazone. IL-6 was measured in the spent culture supernatant using the ELISA method. The *bars* represent the combined mean values (±S.D.) of triplicate samples. *B*: *, p value <0.05.

LPS-induced IκBα phosphorylation as well as IκBα degradation in CMT-93 cells after 1 h of stimulation followed by complete IκBα resynthesis after 2 h of stimulation. Of note, 15d-PGJ_2 and rosiglitazone failed to inhibit LPS-induced IκBα phosphorylation/degradation after 1 h of stimulation. Interestingly and in contrast to rosiglitazone, 15d-PGJ_2 blocked IκBα phosphorylation after 2 h of stimulation. IκBα protein resynthesis was not affected in the presence of any of the two PPARγ agonists.

We next studied LPS-induced nuclear translocation and RelA DNA binding activity in the presence or absence of 15d-PGJ_2 and rosiglitazone in CMT-93 cells. As shown in Fig. 3*C*, whereas LPS-induced RelA DNA binding activity was slightly reduced after 1 h of stimulation, this moderate inhibitory effect was completely abolished after 2 h of stimulation. Rosiglitazone did not affect RelA nuclear translocation or RelA DNA binding activity (Fig. 3*B*).In summary, these results suggest that 15d-PGJ_2 did not affect LPS-induced IκBα degradation, IκBα resynthesis, or RelA nuclear translocation but slightly delayed RelA DNA binding activity in LPS-stimulated IEC.

15d-PGJ_2 Selectively Inhibited IL-6 but Not IκBα Gene Expression in LPS-stimulated IEC—We next sought to investigate the effect of 15d-PGJ_2 and rosiglitazone on LPS-induced IL-6 and IκBα gene expression in CMT-93 cells. Therefore, we stimulated the cells for 12 h with LPS in the presence or absence of 15d-PGJ_2 and rosiglitazone. Interestingly, reverse transcription and real-time PCR analysis revealed that 15d-PGJ_2 but not rosiglitazone inhibited IL-6 mRNA expression in LPS-stimulated CMT-93 cells (Fig. 4*A*). Accordingly, IL-6 protein production was significantly inhibited in the presence of

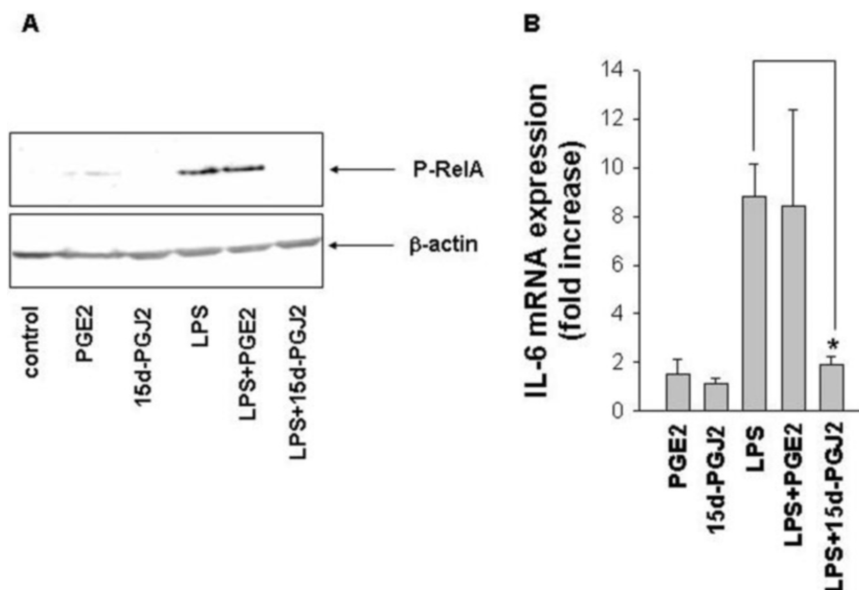

FIG. 5. **15d-PGJ$_2$ but not PGE$_2$ inhibit LPS-induced RelA phosphorylation and IL-6 gene expression.** CMT-93 cells were stimulated with 10 μg/ml LPS from *E. coli* in the presence or absence of 20 μM 15d-PGJ$_2$ and 20 μM PGE$_2$. *A*, total protein was extracted after 1 and 2 h of stimulation, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-RelA and β-actin immunoblotting using ECL technique. *B*, total RNA was extracted after 12 h of stimulation, reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6 and GAPDH. The induction of IL-6 mRNA expression was calculated relative to untreated controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The *bars* represent the combined mean values (±S.D.) of triplicate samples. *, p value <0.05.

15d-PGJ$_2$ but not rosiglitazone (Fig. 4*B*). Consistent with our previous results, IκBα mRNA expression was not affected in the presence of 15d-PGJ$_2$ or rosiglitazone.

Because 15d-PGJ$_2$ but not the synthetic high affinity PPARγ ligand rosiglitazone displayed inhibitory effects on IEC activation, we next transfected CMT-93 cells with PPARγ-specific siRNA oligonucleotides (0.8 μg). Western blot and real-time PCR analysis confirmed PPARγ-specific knock-down (>80%) in CMT-93 cells in the presence of siRNA oligonucleotides. Most importantly, functional analysis in the presence PPARγ-specific siRNA revealed no effect on 15d-PGJ$_2$-mediated inhibition of IL-6 gene expression, confirming PPARγ-independent mechanisms for the inhibitory effects 15d-PGJ$_2$ in LPS-activated IEC (data not shown).

To further elucidate the specificity of 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation, we stimulated CMT-93 cells with LPS in the presence or absence of 15dPGJ$_2$ and PGE$_2$. As shown in Fig. 5, 15d-PGJ$_2$ but not PGE$_2$ blocked LPS-induced RelA phosphorylation (Fig. 5*A*) as well as IL-6 mRNA expression (Fig. 5*B*).

15d-PGJ$_2$-mediated Inhibition of LPS-induced RelA Phosphorylation and IL-6 Gene Expression Is Reversed in the Presence of MEK1 Inhibitor PD98059—The mechanisms of 15d-PGJ$_2$ to inhibit RelA phosphorylation and IL-6 gene expression were independent from the presence of PPARγ. It has been previously shown that 15d-PGJ$_2$ triggers ERK1/2 phosphorylation in murine myoblast cell line C2C12 (48). Based on these results, we next investigated the role of the MAP kinase pathway ERK1/2 to mediate the inhibitory effect of 15d-PGJ$_2$ on LPS-induced IEC activation. Interestingly, 15d-PGJ$_2$ and LPS but not rosiglitazone (data not shown) induced ERK1/2 phosphorylation in CMT-93 cells after 1 h of stimulation, whereas a weak expression of phospho-ERK1/2 remained after 2 h of stimulation in the presence of 15d-PGJ$_2$ but not LPS or LPS/15d-PGJ$_2$ (data not shown). We then used the MEK1 inhibitor PD98059 to inhibit ERK1/2 phosphorylation in CMT-93 cells after the stimulation with LPS for 1 h. Fig. 6*A* shows that the addition of PD98059 inhibited LPS-induced ERK phosphorylation in the presence (*lane 5*) and absence (*lane 7*) of 15d-PGJ$_2$. Interestingly, LPS stimulation in the presence of 15d-PGJ$_2$ further increased ERK1/2 phosphorylation in CMT-93 cells after 1 h of stimulation (*lane 6*). Consistent with our previous results (Figs. 2 and 5), 15d-PGJ$_2$ completely inhibited LPS-induced RelA phosphorylation (*lane 13*). Most importantly, although the presence of PD98059 revealed no effect on LPS-induced RelA phosphorylation (*lane 12*), the presence of the MEK1 inhibitor at least partially reversed 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation (*lane 14*).

To further elucidate the role of the ERK signaling pathway on the inhibitory effects of 15d-PGJ$_2$, we measured LPS-induced IL-6 gene expression in the presence or absence of 15d-PGJ$_2$ after 12 h of stimulation. Real-time RT-PCR analysis revealed that the presence of PD98059 completely reversed 15d-PGJ2-mediated inhibition of LPS-induced IL-6 mRNA expression, whereas PD98059 alone did not affect LPS-induced IL-6 gene expression (Fig. 6*B*). In conclusion, these results suggest that 15d-PGJ$_2$ inhibits LPS-induced RelA phosphorylation as well as IL-6 gene expression in IEC through induction of the ERK-signaling cascade.

15d-PGJ$_2$ Triggers Protein Phosphatase 2A Activity, Which Directly Dephosphorylates RelA—Protein serine/threonine phosphatase activity plays an important role in the regulation of transcription factor activity (24, 25). To further elucidate the role of protein serine/threonine phosphatase activity in 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation and IL-6 gene expression, we used the specific inhibitor calyculin A. Interestingly, the treatment of CMT-93 cells with calyculin A (5 nM) dramatically increased the basal level RelA phosphorylation after 1 h of stimulation (Fig. 7*A*, *lane 2*). The additional treatment of CMT-93 cells with LPS did not further increase RelA phosphorylation (Fig. 7*A*, *lane 5*). In addition and consistent with our previous results, LPS-induced RelA phosphorylation was inhibited in the presence of 15d-PGJ$_2$ (Fig. 7*A*, compare *lane 4* and *6*), and most importantly, calyculin A completely reversed this inhibitory effect of 15d-PGJ$_2$ (Fig. 7*A*, *lane 7*). Total PP2A was similar in all samples, suggesting that PP2A expression remained unchanged during the different treatments. As shown in Fig. 7*B*, LPS-induced IL-6 mRNA expression was significantly increased in the presence of calyculin A. Similar to our previous results, 15d-PGJ$_2$ inhibited LPS-induced IL-6 gene expression. This inhibitory effect was reversed in the presence of calyculin A, suggesting a role of protein phosphatases for the inhibitory effects of 15d-PGJ$_2$ on RelA phosphorylation as well as IL-6 gene expression in CMT-93 cells.

To further specify the role of 15d-PGJ$_2$ in triggering phosphatase activity, we co-immunoprecipitated endogenous phospho-RelA and PP2A after the treatment of CMT-93 cells with

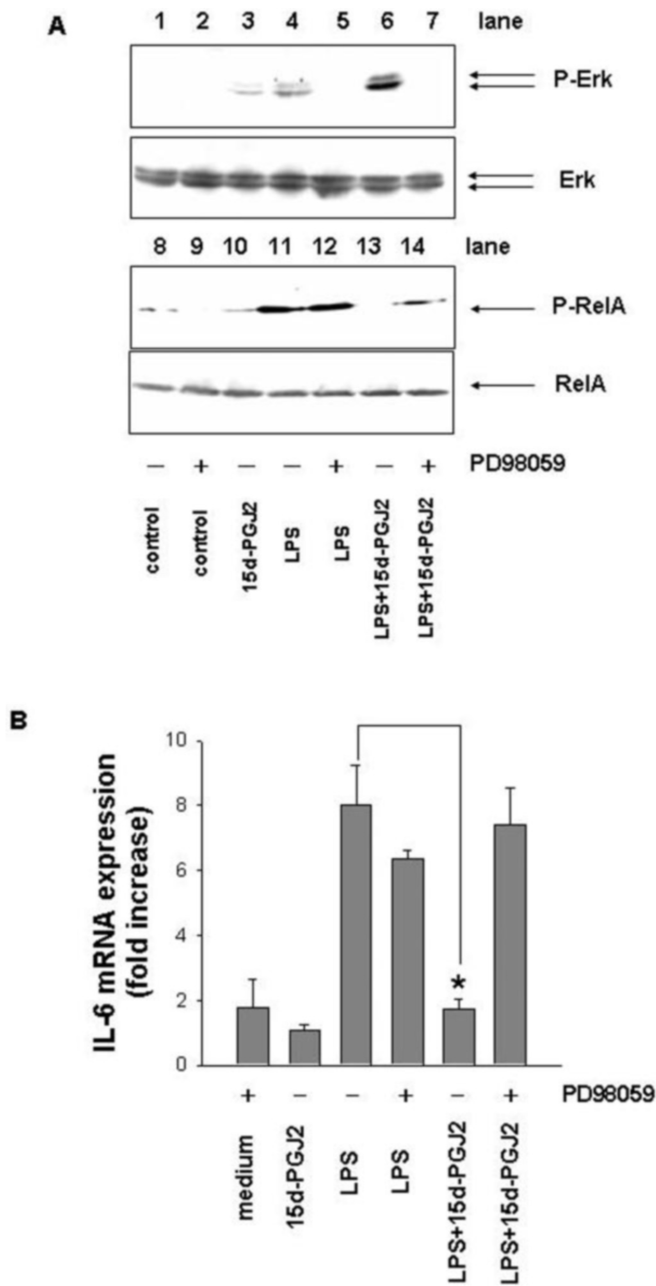

FIG. 6. **The inhibitory effect of 15d-PGJ2 on LPS-induced RelA phosphorylation and IL-6 gene expression was reversed in the presence of PD98059.** CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 1 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 50 μM PD98059. Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-ERK1/2, ERK1/2, phospho-RelA, and RelA immunoblotting using an ECL technique (*A*). CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 12 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 50 μM PD98059. Total RNA was extracted after 12 h of stimulation and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6 and GAPDH. The induction of IL-6 mRNA expression was calculated relative to untreated controls (fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The *bars* represent the combined mean values (±S.D.) of triplicate samples. *, p value <0.05.

LPS and 15d-PGJ$_2$, respectively. Immunoprecipitated phospho-RelA was then co-incubated with PP2A-loaded A/G-agarose beads for additional 1, 2, and 4 h. As shown in Fig. 7*C*, PP2A directly dephosphorylated RelA after 1, 2, and 4 h of co-incubation, suggesting that 15d-PGJ$_2$ triggers PP2A activity in CMT-93 cells. Of note, total RelA and PP2A confirmed equal loading of the samples. In conclusion, we demonstrated that 15d-PGJ$_2$ triggered PP2A activity, which directly dephosphorylated RelA in LPS-stimulated CMT-93 cells.

DISCUSSION

In this study, we demonstrate that 15d-PGJ$_2$ inhibits LPS (*B. vulgatus*)/LPS (*E. coli*)-induced RelA phosphorylation and IL-6 gene expression in the colonic epithelial cell line CMT-93 through induction of the MEK/ERK signaling cascade. Consistent with the transient expression of phospho-RelA in native IEC of *B. vulgatus*-monoassociated Fisher rats, 15d-PGJ$_2$ triggers PP2A activity, which directly dephosphorylates RelA in LPS-stimulated CMT-93 cells.

It appears from several studies that PPARγ agonists including synthetic TZD and 15d-PGJ$_2$ attenuate colonic inflammation in different models of experimental colitis (41, 42, 49–51) and antagonize NF-κB signaling through covalent modification and inhibition of IKKβ in PPARγ-independent mechanisms (37, 44, 45). Indeed, we show PPARγ-independent mechanisms for the inhibitory effect of 15d-PGJ$_2$ in CMT-93 cells using siRNA-mediated knock-down of PPARγ. In addition, several studies now point toward ligand-independent anti-inflammatory effects of PPARγ. For example, studies with mice heterozygous for a deficiency of PPARγ (PPAR$\gamma^{-/+}$) were significantly more susceptible to the development of experimental colitis when compared with wild type mice (42, 50, 51), and accordingly, PPARγ expression in colonic epithelium was substantially reduced in patients with ulcerative colitis (52) as well as dextran sodium sulfate-treated mice (53). Interestingly, *Bacteroides thetaiotaomicron* triggered PPARγ-mediated nuclear export of transcriptionally active RelA and directly abolished *Salmonella enteriditis*-induced inflammatory effects in IEC (54). These results provide compelling evidence that PPARγ plays an important role in the regulation of mucosal inflammation. Taken together, two mechanisms are apparent from these studies. First, PPARγ inhibits inflammatory processes in a ligand-independent manner, and second, PPARγ agonists inhibit NF-κB activity as well as experimental colitis through PPARγ-independent mechanisms. Consistent with previously published results (54), we demonstrate that *B. vulgatus* monoassociation of germ-free Fisher rats triggered persistent nuclear but not cytoplasmic expression of the transcription factor PPARγ in the middle/upper part of the crypts of IEC from large intestinal epithelium. In addition, we show that 15d-PGJ$_2$ but not synthetic TZD inhibits RelA phosphorylation and IL-6 gene expression, confirming previously published results that demonstrate different effects between 15d-PGJ$_2$ and synthetic TZD (38, 55, 56). It remains to be seen whether PPARγ expression is directly involved in the negative regulation of *B. vulgatus*-induced NF-κB signal transduction in the intestinal epithelium.

We also demonstrate that 15d-PGJ$_2$ but not the synthetic high affinity ligand rosiglitazone triggered ERK1/2 phosphorylation in CMT-93 cells and, most importantly, MEK1 inhibitor PD98059 reversed LPS-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells. Gilroy *et al.* (43) show in a model for carrageenan-induced acute inflammation that healing and survival in these mice during the resolution phase of inflammation were associated with increased levels of 15d-PGJ$_2$ and decreased levels of PGE$_2$ (43). Concordently, we show no inhibitory effects of PGE$_2$ on LPS-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells. Furthermore, previous studies demonstrated that 15d-PGJ$_2$, which has no known plasma membrane receptor, activates the MEK/ERK signaling cascade through different mechanisms in various cell types including superoxide anion-dependent induction of the Raf/MEK-signaling pathway in myoblasts (48) as well as induction of the phosphatidylinositol 3-kinase pathway in smooth

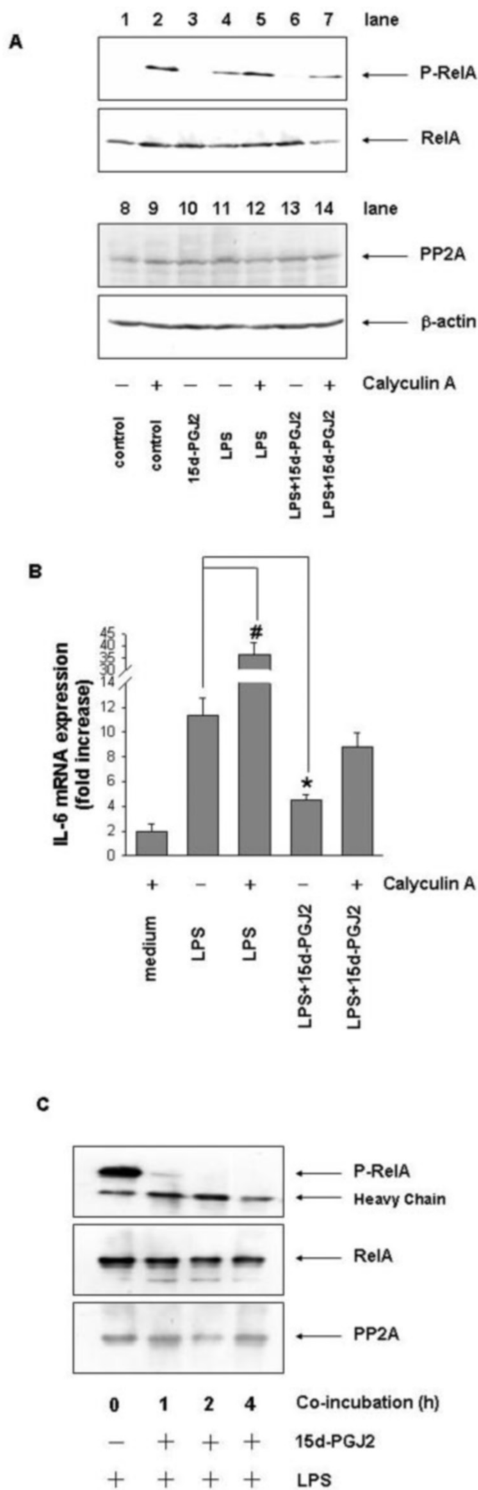

FIG. 7. **Calyculin A reversed the inhibitory effect of 15d-PGJ$_2$ on LPS-induced RelA phosphorylation and IL-6 gene expression in CMT-93 cells; 15d-PGJ$_2$ triggers PP2A activity and directly dephosphorylates RelA.** CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 1 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 5 nM calyculin A. Total protein was extracted, and 20 μg of protein were subjected to SDS-PAGE followed by phospho-RelA, RelA, PP2A, and β-actin immunoblotting using ECL technique (*A*). CMT-93 cells were stimulated with *E. coli* LPS (10 μg/ml) for 12 h in the presence or absence of 20 μM 15d-PGJ$_2$. Where indicated the cells were pretreated for 1 h with 1 nM calyculin A. Total RNA was extracted after 12 h of stimulation and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for murine IL-6 and GAPDH. The induction of IL-6 mRNA expression was calculated relative to untreated controls (-fold increase) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. The *bars* represent the combined mean values ($\pm$S.D.) of triplicate samples. *, reduction; #, increase; p value$<$0.05. *B*, CMT-93 cells were stimulated for 1 h with either *E. coli* LPS (10 μg/ml) or 20 μM 15d-PGJ$_2$. We then immunoprecipitated phospho-RelA and PP2A from lysed cells using A/G-agarose beads. To assay the phosphatase activity, 30 μl of beads from LPS-stimulated cells were co-incubated with 50 μl of beads from 15d-PGJ$_2$-treated cells. Reactions were stopped after 1, 2, and 4 h of incubation at 37 °C with the addition 2× Laemmli sample buffer. Total protein was extracted from beads and subjected to SDS-PAGE followed by phospho-RelA, RelA, and PP2A immunoblotting using ECL technique (*C*).

muscle cells (57). Inhibition of LPS-induced NF-κB activity in the presence of 15d-PGJ$_2$ was demonstrated in native and macrophage cell lines at the level of IKKβ activity, IκBα degradation, and NF-κB DNA binding activity as well as chemokine expression (55, 56, 58). Interestingly, we show that 15d-PGJ$_2$ does not affect LPS-induced IκBα phosphorylation/degradation, IκBα resynthesis, and RelA nuclear translocation in CMT-93 cells. It seems also unlikely that the slight delay in LPS-induced RelA DNA binding activity fully accounts for the complete inhibition of IL-6 gene expression in IEC but, rather, suggests that the 15d-PGJ$_2$-mediated inhibition of RelA phosphorylation directly affects IL-6 gene expression.

Phosphorylation and dephosphorylation of transcription factors regulates their DNA binding properties as well as their transactivating potential, and apparently, complexes containing both protein kinases and phosphoprotein phosphatases are important in maintaining the phosphorylation state of intracellular substrates. PP2A, which regulates a diverse set of cellular processes including signal transduction and transcription, constitutively associates with RelA in the cytoplasm (24, 25). Calyculin A, which is a serine/threonine phosphatase inhibitor, increased base levels of phospho-RelA in untreated CMT-93 cells, suggesting that the basal phosphatase activity has to be relatively active to maintain a low state of phosphorylated RelA in IEC. Upon stimulation of CMT-93 cells with LPS, the constitutive cycle of phosphorylation/dephosphorylation is disrupted presumably through induction of IKKβ, casein kinase, or phosphatidylinositol 3-kinase/Akt activity, resulting in RelA serine phosphorylation (Ser-536). Most importantly, calyculin A, which has higher inhibitory efficiency for PP2A than for PP1 or PP2B, reversed the inhibitory effects of 15d-PGJ$_2$ on LPS-induced RelA dephosphorylation and IL-6 gene expression in CMT-93 cells. Based on these results, we then show for the first time that immunoprecipitated PP2A from 15d-PGJ$_2$-treated CMT-93 cells directly dephosphorylates endogenous RelA (Ser-536) from LPS-treated cells, suggesting that 15d-PGJ$_2$ triggered PP2A activity in epithelial cells. It is not clear whether PP2A binds to the RelA serine phosphorylation sites, but studies on the interaction of PP2A with protein kinase Cζ, casein kinase-2α, and CXCR2 suggest that PP2A binds to other sites than the phosphorylation sites in these proteins (27–29).

Non-pathogenic enteric bacteria play an important role in initiating and perpetuating chronic intestinal inflammation in the susceptible host. It appears from our gnotobiotic studies in normal animals that enteric non-pathogenic bacteria including Gram-negative *B. vulgatus* (17, 22) and Gram-positive *Enterococcus faecalis* (59) trigger transient NF-κB signaling in the intestinal epithelium in normal rats and mice, respectively. In these studies the physiological importance for the induction of protective TGF-β/Smad signaling was clearly demonstrated in native IEC (59). Although TGF-β/Smad signaling blocked non-pathogenic bacteria-induced NF-κB transcriptional activity and IL-6 gene expression in IEC (22), the molecular mechanism for this inhibitory effect was identified at the level of altered

histone acetylation/phosphorylation rather than inhibition of RelA phosphorylation. Based on the results of this study, we may hypothesize that 15d-PGJ$_2$-mediated ERK1/2 signaling contributes to the inhibition of NF-κB-dependent gene expression in IEC through directly acting at the level of RelA phosphorylation by modulating PP2A activity. An attractive hypothesis is that under normal physiological conditions the coordinate induction of various protective mechanisms in IEC including TGF-β/Smad as well as 15d-PGJ$_2$/ERK signaling helps to control NF-κB activity after initial bacterial colonization to maintain normal epithelial cell homeostasis in the interplay with commensal bacteria.

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