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Vitamin D Receptor Negatively Regulates Bacterial-Stimulated NF-κB Activity in Intestine

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Vitamin D receptor (VDR) plays an essential role in gastrointestinal inflammation. Most investigations have focused on the immune response; however, how bacteria regulate VDR and how VDR modulates the nuclear factor (NF)-κB pathway in intestinal epithelial cells remain unexplored. This study investigated the effects of VDR ablation on NF-κB activation in intestinal epithelia and the role of enteric bacteria on VDR expression. We found that $VDR^{-/-}$ mice exhibited a pro-inflammatory bias. After *Salmonella* infection, $VDR^{-/-}$ mice had increased bacterial burden and mortality. Serum interleukin-6 in noninfected $VDR^{+/+}$ mice was undetectable, but was easily detectable in $VDR^{-/-}$ mice. NF-κB p65 formed a complex with VDR in noninfected wild-type mouse intestine. In contrast, deletion of VDR abolished VDR/P65 binding. P65 nuclear translocation occurred in colonic epithelial cells of untreated $VDR^{-/-}$ mice. VDR deletion also elevated NF-κB activity in intestinal epithelia. VDR was localized to the surface epithelia of germ-free mice, but to crypt epithelial cells in conventionalized mice. VDR expression, distribution, transcriptional activity, and target genes were regulated by *Salmonella* stimulation, independent of 1,25-dihydroxyvitamin D3. Our study demonstrates that commensal and pathogenic bacteria directly regulate colonic epithelial VDR expression and location *in vivo*. VDR negatively regulates bacterial-induced intestinal NF-κB activation and attenuates response to infection. Therefore, VDR is an important contributor to intestinal homeostasis and host protection from bacterial invasion and infection. *(Am J Pathol 2010, 177:686–697; DOI: 10.2353/ajpath.2010.090998)*

Vitamin D receptor (VDR) is a nuclear receptor[1] that mediates most known functions of 1,25-dihydroxyvitamin D ($1,25[OH]_2D_3$), the hormonal form of vitamin D. VDR and $1,25(OH)_2D_3$ are involved in calcium homeostasis,[2] electrolyte and blood pressure regulation,[3,4] immune response,[5] and anti-inflammation activity.[6,7] Different ligand shapes of 1, $25(OH)_2D_3$ act through VDR in different cellular locations.[8,9] The target genes of the VDR signal pathway include the enzyme Cyp24 and antimicrobial peptides β-defensin[10,11] and cathelicidin.[12]

Nuclear factor-κB (NF-κB) is a family of transcription factors that play an essential role in innate and adaptive immune responses. NF-κB is active in the nucleus, and its activity is inhibited by the inhibitor of κBα (IκBα).[13] VDR physically interacts with NF-κB subunit p65 in human osteoblasts[14] and mouse embryonic fibroblast cells,[15] but the functional relevance of this VDR/p65 interaction in regulating intestinal inflammation remains unclear.

Human intestinal epithelial cells are constitutively exposed to commensal microbiota and pathogenic bacteria. Enteric commensal bacteria play a crucial role in the pathogenesis of many diseases such as inflammatory bowel diseases.[16–18] and colon cancer.[19,20] Several studies have implicated vitamin D in inflammatory bowel disease (IBD). Low vitamin D levels have been reported in patients with IBD.[21,22] In animal models, $1,25(OH)_2D_3$ suppressed the development of experimental intestinal inflammation.[23,24] Both local and endocrine synthesis of $1,25(OH)_2D_3$ affect murine colitis[25] and VDR status affects the development of murine colitis.[26] VDR mediates T cell homing to the gut.[23] VDR expression is significantly decreased in IBD patients.[22,27] However, the majority of studies of vitamin D, VDR, and inflammation are focused on immunoregulation, with little emphasis on assessing the effects of VDR in intestinal epithelial cells. It is un-

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known how intestinal VDR signaling responds to commensal and pathogenic bacterial stimulation.

In the present study, we hypothesize that *Salmonella typhimurium* infection can induce colonic VDR expression, alter location of VDR *in vivo*, and stimulate VDR expression, transcription, and signaling in colonic epithelial cell lines. We found that mice lacking VDR are in a pre-inflammatory or pro-inflammatory state. Hence, our current study investigated the effect of VDR ablation on NF-κB activation using a VDR knockout mouse model infected with pathogenic *Salmonella* or human commensal *Escherichia coli*. F18. We also examined intestinal VDR expression in normal, germ-free (GF), and conventionalized wild-type mice. Our data demonstrate that intestinal VDR is directly involved in suppression of bacteria-induced NF-κB activation and that commensal bacterial colonization affects intestinal epithelial VDR expression and distribution.

Materials and Methods

Bacterial Strains and Growth Condition

S. typhimurium wild-type ATCC14028, commensal *E. coli* F18, and nonpathogenic *Salmonella* mutant strain PhoP^{c28} were used in this study. Non-agitated micro-aerophilic bacterial cultures were as follows: nonagitated micro-aerophilic bacterial cultures were prepared by inoculation of 10 ml of Luria-Bertani broth with 0.01 ml of a stationary phase culture, followed by overnight incubation (~18 hours) at 37°C, as previously described.[29] Bacterial overnight cultures were concentrated 33-fold in Hanks' balanced salt solution (HBSS) supplemented with 10 mmol/L HEPES, pH 7.4.

Streptomycin Pretreated Mouse Model

Animal experiments were performed by using specific-pathogen-free female C57BL/6 mice (Taconic, Hudson, NY) that were 6 to 7 weeks old[30] and VDR knock out C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) as previously described.[31] The protocol was approved by the University of Rochester University Committee on Animal Resources. Water and food were withdrawn 4 hours before oral gavage with 7.5 mg/mouse of streptomycin (100 μl of sterile solution or 100 μl of sterile water as control). Afterward, animals were supplied with water and food. Twenty hours after streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 1×10^7 colony forming units (CFU) of *S. typhimurium* (100-μl suspension in HBSS) or treated with sterile HBSS (control) by oral gavage as previously described.[32] For the mouse survival experiment, 1×10^8 CFU of *S. typhimurium* was used.

GF and Conventionalized Mouse Colon

GF mice (129/SvEv) were obtained from the Center for Gastrointestinal Biology and Disease Gnotobiotic Core Facility and the National Gnotobiotic Rodent Resource Center, University of North Carolina, Chapel Hill. Mice were analyzed at the University of North Carolina National Gnotobiotic Rodent Resource Center. Sterility of germ-free mice was documented on a monthly basis by fecal Gram stains and aerobic and anaerobic cultures of the feces and bedding. For selected mice, sterility of cecal contents was documented Gram stain and cultures at the time of necropsy. Conventionalized mice were originally GF mice that were infected with a commensal enteric bacteria by orally swabbing fecal slurries from specific pathogen-free (SPF) mice, placing fecal slurries on their food and then housing them in SPF conditions.

Cell Culture

Caco2BBE and HCT116 cells were grown in Dulbecco's Modified Eagle Medium (high glucose, 4.5g/L) containing 5% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin. Monolayers of Caco2BBE cells were grown on permeable supports (0.33 or 4.67 cm^2, 0.4 μm pore. Costar, Cambridge, MA) and used 6 to 14 days after being plated.

Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated from embryonic day 13.5 embryos generated from VDR$^{+/-}$ × VDR$^{+/-}$ mouse breeding as previously described.[15] Cells from each embryo were genotyped by PCR using genomic DNA isolated from the cells. VDR$^{+/-}$ and VDR$^{-/-}$ MEFs were used in experiments after more than 15 passages when they had been immortalized.

Bacterial Colonization in Cultured Cells in Vitro

Human colonic epithelial cells were colonized with equal numbers of the indicated bacteria for 30 minutes, washed with HBSS, and incubated in Dulbecco's Modified Eagle Medium containing gentamicin (500 μg/ml) for the times indicated in our previous studies.[29,33] The first 30-minute incubation allowed bacteria to contact the surface of the epithelial cells and inject the effectors in the host cells. After extensive HBSS washing, the extracellular bacteria were washed away. Incubation with gentamicin inhibited the growth of bacteria. In this way, we focused on the effects of the bacterial effectors injected to the host cells.

Immunoblotting

Mouse colonic epithelial cells were collected by scraping from mouse colon including proximal and distal regions as previously described.[30] Mouse epithelial cells were lysed in lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA pH 8.0, 0.2 mmol/L sodium ortho-vanadate, and protease inhibitor cocktail). MEFs were rinsed twice in ice-cold HBSS, lysed in protein loading buffer (50 mmol/L Tris, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Immunoblot was performed with primary antibodies: anti-VDR, anti-p65,