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BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

The Nucleotide Synthesis Enzyme CAD Inhibits NOD2 Antibacterial Function in Human Intestinal Epithelial Cells

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BACKGROUND & AIMS: Polymorphisms that reduce the function of nucleotide-binding oligomerization domain (NOD)2, a bacterial sensor, have been associated with Crohn's disease (CD). No proteins that regulate NOD2 activity have been identified as selective pharmacologic targets. We sought to discover regulators of NOD2 that might be pharmacologic targets for CD therapies. **METHODS:** Carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD) is an enzyme required for de novo pyrimidine nucleotide synthesis; it was identified as a NOD2-interacting protein by immunoprecipitation-coupled mass spectrometry. CAD expression was assessed in colon tissues from individuals with and without inflammatory bowel disease by immunohistochemistry. The interaction between CAD and NOD2 was assessed in human HCT116 intestinal epithelial cells by immunoprecipitation, immunoblot, reporter gene, and gentamicin protection assays. We also analyzed human cell lines that express variants of NOD2 and the effects of RNA interference, overexpression and CAD inhibitors. **RESULTS:** CAD was identified as a NOD2-interacting protein expressed at increased levels in the intestinal epithelium of patients with CD compared with controls. Overexpression of CAD inhibited NOD2-dependent activation of nuclear factor κB and p38 mitogen-activated protein kinase, as well as intracellular killing of *Salmonella*. Reduction of CAD expression or administration of CAD inhibitors increased NOD2-dependent signaling and antibacterial functions of NOD2 variants that are and are not associated with CD. **CONCLUSIONS: The nucleotide synthesis enzyme CAD is a negative regulator of NOD2. The antibacterial function of NOD2 variants that have been associated with CD increased in response to pharmacologic inhibition of CAD. CAD is a potential therapeutic target for CD.**

Keywords: NLR; Innate Immunity; IBD; PALA.

Crohn's disease (CD) is a recurrent and often debilitating inflammatory bowel disease that affects more than 500,000 individuals in the United States. Although the cause of CD is currently unknown, both genetic components as well as environmental factors are required for disease development.[1,2] Additional lines of evidence show a key role for an altered immune response to microbial factors in the pathogenesis of CD.[3,4] Individuals with CD have severe abnormalities in acute inflammatory immune responses to bacteria.[5] In addition, diversion of the fecal stream or microflora manipulation by antibiotics or diet can increase disease remission, supporting the idea that CD depends on the presence and composition of the gut microflora.[4,6–11] These findings suggest that the chronic inflammation seen in CD results from an inappropriate immune response to bacteria.

NOD2 was the first CD susceptibility gene identified and codes for one member of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family of intracellular pattern recognition molecules.[3] NLRs induce inflammatory and antimicrobial immune responses to either bacteria/bacterial-derived components or cell "danger signals" released from injured or necrotic cells.[12] NOD2 detects bacteria by recognizing a specific component of peptidoglycan called muramyl dipeptide (MDP), which is generated during bacterial infection. MDP is a common component of peptidoglycan from both Gram-positive and Gram-negative bacteria, indicating that NOD2 is a sensor of a broad range of bacteria.

Abbreviations used in this paper: CAD, carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase; CARD, caspase recruitment domain; CPSII, carbamoyl phosphate synthetase II; LRR, leucine-rich repeat; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; NF-κB, nuclear factor κB; NLR, nucleotide-binding, oligomerization domain-like receptor; NOD2, nucleotide-binding oligomerization domain 2; NP-40, Nonidet P-40; PALA, *N*-phosphonacetyl-L-aspartate; RNAi, RNA interference.

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NOD2 genetic variants have been repeatedly linked to CD.[3] The 3 main risk variants of *NOD2* include 2 missense mutations, R702W and G908R, and one frameshift mutation, L1007fsinsC (L1007fs). Although some controversy remains about the functional effects of these *NOD2* mutations, most studies indicate that these CD-associated variants have defects in inflammatory signaling and bacterial killing in response to MDP.[13] The exact mechanism by which a loss of NOD2-dependent responses leads to an inflammatory disease is unclear. Decreased NOD2 results in an increased bacterial load and shifts in bacterial species in the intestine[14] and impairs antibacterial responses.[15,16] Animal studies also show a protective role for NOD2-dependent responses in colitis.[17,18] Therefore, it appears that the down-regulation of NOD2 function is an important contributor to the pathogenesis of CD.

The importance of NOD2 function to maintain mucosal health has led to the identification of specific regulators of NOD2. Although these proteins include both positive (XIAP, GRIM19, and CARD9) and negative (Erbin, TRAF4, NLRC4, CARD8, β-PIX, Centaurin β1, and Rac-1) regulators,[19–28] none of these regulators are selective pharmacologic targets for modulation of NOD2 function. These proteins act as protein scaffolds, integrators of cellular responses, or actin cytoskeleton modulators. Therefore, we performed immunoprecipitation-coupled mass spectrometry to identify additional regulators of NOD2 with the goal of identifying proteins that could be pharmacologically targeted to enhance NOD2 function. From these studies, we identified carbamoyl phosphate synthetase/aspartate transcarbamylase/dihydroorotase (CAD), an enzyme essential for de novo pyrimidine synthesis,[29] as a novel negative regulator of NOD2. Our studies show that modulation of CAD expression levels or enzyme activity dramatically affects NOD2 activity. In addition, we found that treatment with CAD inhibitors enhances the function of both wild-type NOD2 and CD-associated defective NOD2 variants. Our findings suggest that CAD may be a novel therapeutic target for CD.

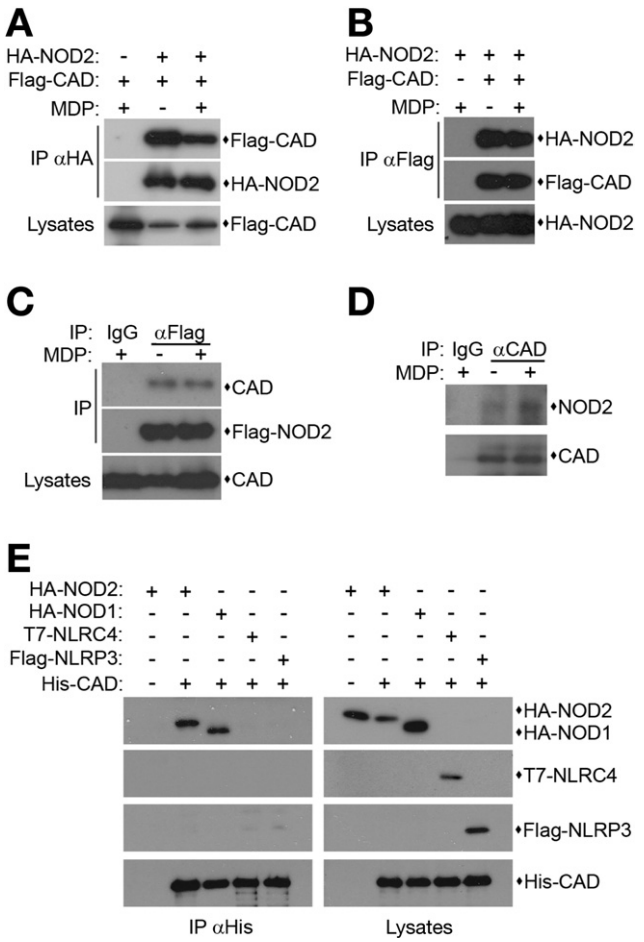

Figure 1. CAD is a NOD2-interacting protein. (*A*) HEK293T cells were transfected with Flag-CAD and HA-NOD2 constructs, MDP stimulated (100 ng/mL for 30 minutes) and lysates were immunoprecipitated with HA antibody, followed by immunoblot. (*B*) Same as in *A* except immunoprecipitation with Flag antibody. (*C*) 293:Flag-NOD2 cells were treated with MDP (100 ng/mL for 30 minutes) and lysates immunoprecipitated with Flag antibody or rabbit immunoglobulin G (IgG), followed by immunoblot. (*D*) HCT116 cells were MDP stimulated (10 μg/mL for 30 minutes) and lysates immunoprecipitated with CAD antibody or rabbit IgG, followed by immunoblot. (*E*) HEK293T cells were transfected with the indicated expression constructs and lysates immunoprecipitated with His antibody, followed by immunoblot.

Materials and Methods

Cell Lines

HCT116, HEK293T, 293:pMXp, and 293:Flag-NOD2 cell lines were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Lonza, Allendale, NJ). The 293:pMXp and 293:Flag-NOD2 lines were generated by retroviral infection of HEK293 cells and antibiotic selection. The 293:Flag-NOD2 subclones were isolated and screened for low levels of Flag-NOD2 expression by immunoblot.

Immunoprecipitation-Coupled Mass Spectrometry Screen

The 293:pMXp and 293:Flag-NOD2 cell lines were stimulated with Ac-(6-O-stearoyl)-muramyl-Ala-D-Glu-NH_2 (1 μg/mL for 1 hour; Bachem, Torrance, CA) and then lysed in Nonidet P-40 (NP-40) lysis buffer (Phosphatase Inhibitor Cocktail I, Sigma (St. Louis, MO); 10 mmol/L HEPES, pH 7.4, 142 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L ethylene glycol-bis[β-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid, 0.2% NP-40, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 100 μg/mL phenylmethylsulfonyl fluoride). Lysates were precleared with mouse immunoglobulin G–agarose (Sigma) and then incubated with Flag M2-agarose (Sigma) and immunocomplexes washed with NP-40 lysis buffer, BC+ buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol-bis[β-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid, 0.2% NP-40), and Tris-buffered saline (25 mmol/L Tris, pH 7.5, 146 mmol/L NaCl, 8 mmol/L KCl) and then eluted with Flag peptide. Coimmunoprecipitation of RIP2 was confirmed by immunoblot of an aliquot of the eluate before analysis (Supplementary Figure 1) Eluates were acetone precipitated and separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis before trypsin digestion and analysis by liquid chromatography–coupled tandem mass spectrometry.