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

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Abstract

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 κ B and p38 mitogen-activated protein kinase, as well as intracellular killing of *Salmonella*. Reduction of CAD expression or

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2012.02.040>.

Conflicts of interest

The authors disclose no conflicts.

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.

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 intestine¹⁴ 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 downregulation 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 β 1, 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.

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-NH₂ (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 MgCl₂, 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.

Immunoprecipitation and Immunoblot

Cells were transfected with expression plasmids by calcium phosphate and lysates immunoprecipitated and analyzed by immunoblot as previously described.²⁵ For phospho-specific immunoblots, cells were lysed in NP-40 lysis or ginger buffer (312 mmol/L Tris, pH 6.8, 25% glycerol, 5% sodium dodecyl sulfate, 1.7 mol/L β-mercaptoethanol).

Immunohistochemistry

Deidentified colonic tissue taken from individuals undergoing colonic resection was obtained from the Cooperative Human Tissue Network (Supplementary Table 1), fixed in Histochoice (Amresco, Solon, OH), and embedded in paraffin. Antibody staining was performed on serial 4-µm sections after microwave-mediated antigen retrieval (30 minutes, medium power) in 10 mmol/L citrate buffer, pH 6.0. Peroxidases were quenched with 3% H₂O₂ (10 minutes). Slides were blocked in 5% bovine serum albumin (30 minutes) followed by incubation overnight at 4°C with anti-CAD antibody (1:200; Novus Biologicals, Littleton, CO). Sections were incubated with horseradish peroxidase-conjugated secondary antibodies (1 hour at room temperature) followed by 5-minute staining with 3,3'-diaminobenzidine and hematoxylin blue. Images (40×) of at least 4 separate fields/individual were taken on an Olympus BX-41 microscope equipped with a Q-Color3 camera (Olympus, Center Valley, PA). Staining intensity (pixels/µm) was determined using Image Pro Plus

v7.0 software (Media Cybernetics, Bethesda, MD). Image analysis was restricted to the mucosa, because this is where the most dramatic change in CAD expression was observed between CD patient tissue and controls.

Luciferase Reporter Assays

Nuclear factor κ B (NF- κ B) luciferase reporter assays were performed as previously described.³⁰ Mitogen-activated protein kinase (MAPK) p38 activity was measured using the PathDetect CHOP *trans*-Reporting System (Invitrogen) in NOD2-expressing HEK293T cells transfected in triplicate using Polyfect (Qiagen, Valencia, CA). Cells were stimulated with MDP (100 ng/mL for 16 hours) and assayed using the Luciferase Reporter Assay System (Promega, Madison, WI) and for β -galactosidase activity.³⁰ Luciferase values were normalized to β -galactosidase absorbance, and statistical significance between groups was determined by unpaired 2-tailed *t* test. Differences were considered significant when $P < .05$.

Gentamicin Protection Assay

Intracellular killing of *Salmonella enterica* serovar *typhimurium* SL1344 was assessed by gentamicin protection assay as previously described.³⁰

Results

CAD Is a Novel NOD2 Interacting Protein

Due to the link between diminished NOD2 function and the pathogenesis of CD, we performed an immunoprecipitation-coupled mass spectrometry screen to identify novel NOD2 regulatory proteins. NOD2 protein complexes were immunoprecipitated from lysates of MDP-stimulated HEK293 cells stably expressing low levels of Flag-tagged NOD2 to mimic endogenous expression (293:Flag-NOD2). Protein complexes were eluted from the beads by incubation with Flag peptide and identified by liquid chromatography–coupled tandem mass spectrometry (Supplementary Figure 1A). As a control for nonspecific protein binding, lysates from MDP-stimulated 293:pMXp cells were processed in the same manner and proteins unique to the 293:Flag-NOD2 prep were considered candidates. CAD, an essential *de novo* pyrimidine nucleotide synthesis enzyme,²⁹ was one of the novel NOD2-interacting proteins identified. Additionally, several other candidate proteins involved in nucleotide synthesis copurified with NOD2 (Supplementary Figure 1B). Currently, immunomodulators that affect nucleotide synthesis (azathioprine and 6-mercaptopurine) are used therapeutically for CD,³¹ suggesting that the interaction of NOD2 with nucleotide synthesis enzymes may have a disease-relevant relationship. Consistent identification of CAD in multiple purifications drove us to focus on the functional interaction of CAD and NOD2.

Because MDP stimulation of NOD2 results in the formation of a large protein complex to initiate inflammatory and antibacterial responses,¹² we examined whether MDP stimulation was required for the interaction of CAD with NOD2. First, we assessed the interaction of these 2 proteins in HEK293T cells overexpressing epitope-tagged versions of CAD (Flag-CAD) and NOD2 (HA-NOD2). Lysates from untreated or MDP-stimulated cells were immunoprecipitated and analyzed by immunoblot. Confirming the results of the screen, we observed Flag-CAD coimmunoprecipitating with HA-NOD2, as well as the converse interaction of HA-NOD2 coimmunoprecipitating Flag-CAD, in both basal and MDP-stimulated conditions (Figure 1A and B). Next, we examined this interaction in 293:Flag-NOD2 cells and observed coimmunoprecipitation of the endogenous CAD protein with Flag-NOD2 in the presence and absence of MDP stimulation (Figure 1C). Finally, we asked whether endogenous CAD and NOD2 proteins interact in HCT116 human intestinal epithelial cells. We found that endogenous CAD immunoprecipitated endogenous NOD2 in

both untreated and MDP-stimulated cells (Figure 1D). Additionally, we examined whether CAD could interact with other members of the NLR family or if this interaction was specific to NOD2. Epitope-tagged NLR family members NOD1, NOD2, NLRC4, and NLRP3 were overexpressed with His-tagged CAD in HEK293T cells and interaction was assessed by coimmunoprecipitation. Only NOD1 and NOD2 coimmunoprecipitated with CAD (Figure 1E), suggesting that CAD may interact with a specific subset of intracellular bacterial sensors. These findings show that CAD is a novel NOD2-interacting protein that interacts with NOD2 in an MDP-independent manner.

CAD Protein Expression in Intestinal Epithelial Cells Is Increased in CD

NOD2 has antibacterial roles in both epithelial and monocytic-derived cells.^{13,26,32–34} We examined CAD protein expression in the intestine by immunohistochemistry of colon tissue sections obtained from colonic resections of patients with and without inflammatory bowel disease (Supplementary Table 1 and Supplementary Figure 2A). In control individuals, we observed low levels of CAD protein in the epithelium, as well as in lamina propria mononuclear cells (Figure 2A). In comparison, overall mucosal CAD expression was increased in patients with CD but not in individuals with ulcerative colitis (Figure 2B and C). Semiquantitative densitometric analysis of the average CAD staining intensity per cell confirmed this dramatic change of CAD expression in individuals with CD (Figure 2E) and determined that the largest changes occur in the epithelium (Figure 2F). Interestingly, NOD2 expression has also been reported to be increased in the epithelium of patients with CD.^{33–35} To determine whether CAD expression is regulated by NOD2 activity, we measured CAD expression before and after either MDP or tumor necrosis factor α stimulation of HCT116 cells by quantitative real-time polymerase chain reaction. Neither MDP nor tumor necrosis factor α significantly changed CAD messenger RNA expression (<1.5-fold increase), suggesting that the changes in CAD expression observed in colonic epithelial cells are independent of NOD2 signaling (Supplementary Figure 3). These findings indicate that CAD is normally expressed at low levels in cells of the intestinal mucosa and that individuals with CD have significantly increased CAD expression, which is most dramatically altered in the intestinal epithelium.

CAD Is a Negative Regulator of NOD2 Signaling and Bacterial Killing

Bacterial infection or MDP stimulates NOD2-dependent signaling, which results in activation of NF- κ B and MAPKs, p38, Jnk, and Erk1/2, promoting inflammation and clearance of intracellular bacteria.³⁶ To determine the effect of the interaction of CAD and NOD2 on NOD2 function, we manipulated CAD expression levels and assessed the effect on NOD2-dependent signaling and bacterial killing. We assessed NF- κ B activation by reporter assays in NOD2-expressing HEK293T cells. HEK293T cells do not express detectable levels of NLRs and only become responsive to bacterial ligands (such as MDP) when the NLR is ectopically expressed.³⁷ Transfection of increasing amounts of CAD expression plasmid resulted in a dose-dependent decrease of NOD2-dependent NF- κ B reporter gene activity stimulated by MDP (Figure 3A). The converse was also observed when CAD expression was decreased by RNA interference (RNAi) (Figure 3B). Additionally, MDP-stimulated p38 activity was inhibited by overexpression of CAD in p38 reporter assays (Figure 3C). Similar effects were observed when NOD1 activity was assessed in these assays (Figure 3E); however, CAD does not appear to be a global regulator of NF- κ B activity because tumor necrosis factor α -stimulated NF- κ B activity was not repressed by overexpression of CAD (Figure 3F).

CAD catalyzes the first 3 steps for conversion of glutamine into the pyrimidines uridine triphosphate and cytidine triphosphate, which are used in the formation of RNA, DNA, uridine 5'-diphosphate sugars, and phospholipids required for cellular proliferation.²⁹ CAD

enzymatic activity and expression level are tightly regulated by cellular proliferative rate and differentiation state.²⁹ We observed in immunoblots of HCT116 cell lysates that CAD protein expression varied widely depending on culture confluency, with actively proliferating cells (low density) having higher CAD expression levels than cells with a lower proliferation rate due to contact inhibition (high density) (Figure 3D). We exploited this endogenous regulation of CAD expression to further examine the modulation of NOD2 activity by CAD expression levels in an unmanipulated system. HCT116 cells were plated at high and low density and MDP stimulated, then NOD2-dependent signaling was assessed by phospho-specific immunoblots (Figure 3D). In cells plated at low confluency (high CAD expression), peak p65 NF- κ B and p38 MAPK phosphorylation occurred after 2 hours of MDP stimulation. However, in confluent cultures (low CAD expression), MDP-stimulated signaling was not only increased but peaked earlier (1.5 hours). These results show that CAD is a negative regulator of NOD2 signaling.

To examine the role of CAD on antibacterial function mediated by NOD2, we measured the effect of CAD expression level on the killing of an intracellular bacterial pathogen, *Salmonella typhimurium*, in gentamicin protection assays using HCT116 cells. Although MDP is released during the normal course of bacterial infection, exogenous MDP stimulation during infection results in enhanced bacterial killing, an effect that is lost in cells lacking functional NOD2.^{30,32,38–41} To determine whether CAD expression levels affect this NOD2-dependent enhancement of bacterial killing, we increased CAD expression levels in HCT116 cells by transfection and found that overexpression of CAD blocks MDP-enhanced *Salmonella* killing (Figure 4A). Interestingly, when CAD expression is decreased by RNAi, *Salmonella* killing was increased in the absence of exogenous MDP (Figure 4B). This increased antibacterial function was NOD2 dependent, because blocking NOD2 function by transfection of a dominant negative NOD2 construct abolished this effect (Figure 4C). These findings indicate that the regulation of NOD2 function by CAD has a dramatic effect on clearance of the intracellular pathogen *Salmonella*.

Specific Regions Within NOD2 and CAD Are Required for Interaction and NOD2 Inhibition

Delineation of the regions within CAD and NOD2 required for interaction could provide clues as to the molecular mechanisms of CAD inhibition of NOD2. NOD2 has 3 distinct structural regions: (1) carboxylterminal leucine-rich repeats (LRRs) required for sensing of MDP, (2) a central NOD involved in formation of active oligomers, and (3) amino-terminal caspase recruitment domains (CARDs) that recruit downstream signal transduction molecules.¹³ CAD is composed of 3 separate enzymes: carbamoyl phosphate synthetase II (CPSII), aspartate transcarbamylase, and dihydroorotase.²⁹

To define the specific regions of interaction in both NOD2 and CAD, we assessed the coimmunoprecipitation of deletion constructs of either HA-NOD2 or Flag-CAD with full-length proteins in HEK293T cells. First, we determined that only NOD2 constructs that contained the CARDs (CARDs and Δ LRR constructs) coimmunoprecipitated with CAD, whereas the NOD or LRRs alone did not bind CAD (Figure 5A). Next, we observed that CPSII was sufficient to coimmunoprecipitate NOD2, with no consistent changes in binding affinity with MDP stimulation (Figure 5B). These results indicate that these 2 proteins interact via the CARDs of NOD2 and the CPSII enzyme of CAD.

To determine whether the interaction of NOD2 with CPSII was sufficient for inhibition of NOD2, we tested the effect of CPSII overexpression on NOD2 function by NF- κ B and p38 reporter assays. We observed that both signaling pathways were inhibited by either full-length CAD or CPSII (Figure 5C and D). In addition, CPSII expression was sufficient to block MDP-enhanced *Salmonella* killing in HCT116 cells (Figure 5E). These findings indicate that the CPSII region of CAD mediates both interaction and repression of NOD2.

Pharmacologic Inhibitors of CAD Enhance NOD2 Antibacterial Function

The mechanism by which CAD inhibits NOD2 potentially includes enzymatic as well as physical mechanisms. To better understand the relative contributions of these mechanisms, we tested the effects of 2 pharmacologic inhibitors of CAD on NOD2-dependent bacterial killing. These CAD inhibitors included acivicin (L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), which interferes with the binding of glutamine to CPSII, and *N*-phosphonacetyl-L-aspartate (PALA), a highly specific, transition-state inhibitor of aspartate transcarbamylase^{42,43} (Figure 6A). When HCT116 cells were pretreated with either CAD inhibitor, we observed increased intracellular *Salmonella* killing (Figure 6B). When examined further, we found that RNAi-mediated knockdown of NOD2 expression blocked the enhancement of bacterial killing by PALA (Figure 6C), indicating that this effect is mediated by NOD2. Our data also suggest that the effect of PALA on NOD2-dependent bacterial killing is independent of pyrimidine depletion, because these effects occurred rapidly (~1 hour) and at low doses (3 μmol/L) in the presence of nucleotide precursors that could be used by the salvage pathway to maintain cellular pyrimidine levels (Supplementary Figure 4).

Complementing these results, we observed that pretreatment of HCT116 cells with PALA increased MDP-stimulated NF-κB and p38 MAPK activation. Pretreatment of HCT116 cells with PALA not only increased the MDP-stimulated phosphorylation of the NF-κB subunit p65 and p38 MAPK but also caused earlier peak activation of these molecules (Figure 6D and E). Similar enhancement of NOD2 signaling with acivicin treatment was also observed (Supplementary Figure 5). However, only a fraction of the acivicin-enhanced bacterial killing was blocked by NOD2 RNAi (Supplementary Figure 5), suggesting that additional antibacterial pathways are modulated by this broader-spectrum inhibitor. These results show that pharmacologic inhibition of CAD increases NOD2-dependent signaling and bacterial killing.

Inhibition of CAD Enhances the Function of CD-Associated NOD2 Variants

Because reduction of CAD expression or treatment with CAD inhibitors dramatically increased normal NOD2 function, we investigated whether the impaired function of NOD2 risk variants could be improved by reducing CAD expression or activity. We examined whether CAD interacted with NOD2 risk variants to predict whether CAD could regulate their function. Flag-CAD was immunoprecipitated from HEK293T cells expressing HA-NOD2 or HA-NOD2 mutants and interaction detected by immunoblot. No significant differences in CAD binding to the NOD2 risk variants as compared with wild-type NOD2 were observed (Figure 7A). These results indicate that the CD-associated NOD2 mutations do not disrupt interaction with CAD.

Next, we assessed what effect CAD inhibition had on the function of NOD2 risk variants. The 3 major NOD2 risk variants have varying degrees of functional impairment, as shown by reduced NF-κB activation and bacterial killing in response to MDP stimulation (Figure 7B and C). RNAi-mediated knockdown of CAD expression in HEK293T cells expressing NOD2 or the NOD2 risk variants resulted in increased MDP-stimulated NF-κB reporter gene activity for all NOD2 constructs (Figure 7B). Additionally, in CAD RNAi-treated cells, the amount of bacterial killing by NOD2 CD-associated variants was equivalent to that observed for wild-type NOD2 (Figure 7D). Importantly, we observed a similar increase in bacterial killing by NOD2 variants in cells pretreated with the CAD inhibitor PALA (Figure 7E). These increases in bacterial killing were dependent on NOD2 expression, because manipulation of CAD expression by RNAi or CAD activity by PALA treatment did not enhance bacterial killing in vector transfected cells. These findings indicate that the

antibacterial function of CD-associated NOD2 variants can be enhanced by either decreasing CAD expression levels or pharmacologically inhibiting CAD activity.

Discussion

CD-associated *NOD2* variants reduce NOD2 function and contribute to the development of CD.³ Impairments in NF- κ B activation, proinflammatory cytokine secretion, α -defensin production, and bacterial killing have been observed in patients with CD who have disease-associated *NOD2* variants.¹³ Loss of NOD2 is associated with increases in intestinal bacterial load, as well as specific changes in microflora composition.¹⁴ In addition, NOD2 may be a negative regulator of other bacterial sensor proteins¹⁸ and contribute to dampening of inflammatory responses to the microflora. Taken together, these NOD2 defects are postulated to lead to CD through ineffective bacterial clearance resulting in chronic infection, as well as inappropriate inflammatory responses to an altered microflora.

Experimental colitis models suggest that activation of NOD2 is protective and may be beneficial for patients with CD.^{17,18} However, therapies specifically enhancing NOD2 function do not currently exist because known NOD2 regulatory proteins either lack enzymatic function or are not selective pharmacologic targets.^{19–27} In our studies, we identified the de novo pyrimidine synthesis enzyme CAD as a novel negative regulator of NOD2 signaling and antibacterial function in intestinal epithelial cells. Although the precise molecular mechanism by which CAD exerts its inhibitory effect on NOD2 function requires more study, our results showing (1) a pharmacologic block of CAD-mediated repression of NOD2 and (2) the MDP-independent association of CAD with NOD2 suggest that the enzymatic activity of CAD is an important contributor to NOD2 inhibition. Our findings show a new role for CAD in host defense and suggest that this may be a target for immune modulation.

CAD is a target of specific drugs already tested in clinical trials,^{44,45} and we show that treatment of epithelial cells with these pharmacologic inhibitors increases NOD2 signaling and bacterial killing. How inhibition of CAD activity improves the function of these variants is still under investigation. One possibility is that CAD inhibition increases the function of other pattern recognition molecules, such as NOD1, as a compensatory response. However, we observed in our experiments that NOD2 is required for enhancement of bacterial killing by either reducing CAD expression or treatment with PALA (Figures 4, 6, and 7). Biochemical studies of NLRs propose that the LRRs fold back over the NOD region to inhibit activation, because deletion of this region results in constitutive activity.^{46–48} If CAD is participating in maintenance of NOD2 in an inactive state, then conformational changes induced in CAD by the binding of PALA or a loss of an inhibitory enzymatic product of CAD could result in a more open conformation of NOD2 that is more sensitive to stimulation by MDP. Interestingly, the CAD inhibitor PALA also enhances the antibacterial function of disease-associated NOD2 variants. Because the CD-associated variants of NOD2 are localized either in or near the LRRs,¹³ one result of these mutations could be the stabilization of an inactive conformation, resulting in a NOD2 more refractory to activation by MDP. We postulate that by releasing the inhibitory effects of CAD on these NOD2 risk variants, their conformation could shift to a state closer to the normal structure, which is more sensitive to MDP stimulation.

Increasing NOD2 function has clear clinical implications for *NOD2* variant-associated CD but may also be beneficial to patients without a *NOD2* risk genotype. *NOD2* variants account for only a fraction of all cases of CD (10%–20% of white subjects are homozygous or compound heterozygous carriers of *NOD2* variants).³ A recent genome-wide meta-analysis identified 71 CD-associated loci, expanding the view of genetic contributions to the

pathogenesis of CD from dysfunction of a single gene to the concept that several CD risk genes may function in a common response pathway altered in disease.^{49,50} This is supported by studies implicating autophagy as one central mechanism altered in CD, which is affected by both *NOD2* and *ATG16L1* CD-associated variants.^{30,51,52} In addition, a recent study suggests that impaired *NOD2* function may be a common feature of patients with CD, even those with a normal *NOD2* genotype.⁵³ One potential contributing factor to this nongenetic repression of *NOD2* function could be the increased CAD expression we observed in the intestinal mucosa of patients with CD. Our findings show a new role for CAD in host defense and suggest that CAD could be a novel therapeutic target to enhance *NOD2* function in patients with CD, warranting further investigation in animal models of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 κ B
NLR	nucleotide-binding, oligomerization domain-like receptor
NOD2	nucleotide-binding oligomerization domain 2
NP-40	Nonidet P-40
PALA	<i>N</i> -phosphonacetyl- <i>L</i> -aspartate
RNAi	RNA interference.

References

1. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol.* 2010; 28:573–621. [PubMed: 20192811]

2. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007; 448:427–434. [PubMed: 17653185]
3. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol*. 2008; 8:458–466. [PubMed: 18500230]
4. Marks DJ, Segal AW. Innate immunity in inflammatory bowel disease: a disease hypothesis. *J Pathol*. 2008; 214:260–266. [PubMed: 18161747]
5. Marks DJ, Harbord MW, MacAllister R, et al. Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet*. 2006; 367:668–678. [PubMed: 16503465]
6. D'Haens GR, Geboes K, Peeters M, et al. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology*. 1998; 114:262–267. [PubMed: 9453485]
7. O'Morain C, Segal AW, Levi AJ. Elemental diet as primary treatment of acute Crohn's disease: a controlled trial. *Br Med J (Clin Res Ed)*. 1984; 288:1859–1862.
8. Rutgeerts P, Geboes K, Peeters M, et al. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet*. 1991; 338:771–774. [PubMed: 1681159]
9. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology*. 2004; 126:1620–1633. [PubMed: 15168372]
10. Saverymutter S, Hodgson HJ, Chadwick VS. Controlled trial comparing prednisolone with an elemental diet plus non-absorbable antibiotics in active Crohn's disease. *Gut*. 1985; 26:994–998. [PubMed: 3902590]
11. Winslet MC, Allan A, Poxon V, et al. Faecal diversion for Crohn's colitis: a model to study the role of the faecal stream in the inflammatory process. *Gut*. 1994; 35:236–242. [PubMed: 8307475]
12. Philpott DJ, Girardin SE. Nod-like receptors: sentinels at host membranes. *Curr Opin Immunol*. 2010; 22:428–434. [PubMed: 20605429]
13. Abraham C, Cho JH. Functional consequences of NOD2 (CARD15) mutations. *Inflamm Bowel Dis*. 2006; 12:641–650. [PubMed: 16804402]
14. Petnicki-Ocwieja T, Hrcir T, Liu YJ, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci U S A*. 2009; 106:15813–15818. [PubMed: 19805227]
15. Biswas A, Liu YJ, Hao L, et al. Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum. *Proc Natl Acad Sci U S A*. 2010; 107:14739–14744. [PubMed: 20679225]
16. Kobayashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*. 2005; 307:731–734. [PubMed: 15692051]
17. Watanabe T, Asano N, Murray PJ, et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J Clin Invest*. 2008; 118:545–559. [PubMed: 18188453]
18. Yang Z, Fuss IJ, Watanabe T, et al. NOD2 transgenic mice exhibit enhanced MDP-mediated down-regulation of TLR2 responses and resistance to colitis induction. *Gastroenterology*. 2007; 133:1510–1521. [PubMed: 17915219]
19. Barnich N, Hisamatsu T, Aguirre JE, et al. GRIM-19 interacts with nucleotide oligomerization domain 2 and serves as downstream effector of anti-bacterial function in intestinal epithelial cells. *J Biol Chem*. 2005; 280:19021–19026. [PubMed: 15753091]
20. Bauler LD, Duckett CS, O'Riordan MX. XIAP regulates cytosol-specific innate immunity to *Listeria* infection. *PLoS Pathog*. 2008; 4:e1000142. [PubMed: 18769721]
21. Damiano JS, Oliveira V, Welsh K, et al. Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. *Biochem J*. 2004; 381:213–219. [PubMed: 15107016]
22. Eitel J, Krull M, Hocke AC, et al. Beta-PIX and Rac1 GTPase mediate trafficking and negative regulation of NOD2. *J Immunol*. 2008; 181:2664–2671. [PubMed: 18684957]
23. Hsu YM, Zhang Y, You Y, et al. The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens. *Nat Immunol*. 2007; 8:198–205. [PubMed: 17187069]

24. Kufer TA, Kremmer E, Banks DJ, et al. Role for erbin in bacterial activation of Nod2. *Infect Immun*. 2006; 74:3115–3124. [PubMed: 16714539]
25. McDonald C, Chen FF, Ollendorff V, et al. A role for Erbin in the regulation of Nod2-dependent NF-kappaB signaling. *J Biol Chem*. 2005; 280:40301–40309. [PubMed: 16203728]
26. von Kampen O, Lipinski S, Till A, et al. Caspase recruitment domain-containing protein 8 (CARD8) negatively regulates NOD2-mediated signaling. *J Biol Chem*. 2010; 285:19921–19926. [PubMed: 20385562]
27. Yamamoto-Furusho JK, Barnich N, Xavier R, et al. Centaurin beta1 down-regulates nucleotide-binding oligomerization domains 1- and 2-dependent NF-kappaB activation. *J Biol Chem*. 2006; 281:36060–36070. [PubMed: 17005562]
28. Marinis JM, Homer CR, McDonald C, et al. A novel motif in the Crohn's disease susceptibility protein, NOD2, allows TRAF4 to down-regulate innate immune responses. *J Biol Chem*. 2011; 286:1938–1950. [PubMed: 21097508]
29. Huang M, Graves LM. De novo synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways. *Cell Mol Life Sci*. 2003; 60:321–336. [PubMed: 12678497]
30. Homer CR, Richmond AL, Rebert NA, et al. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology*. 2010; 139:1630–1641. 1641. e1-2. [PubMed: 20637199]
31. Engel MA, Neurath MF. New pathophysiological insights and modern treatment of IBD. *J Gastroenterol*. 2010; 45:571–583. [PubMed: 20213337]
32. Hisamatsu T, Suzuki M, Reinecker HC, et al. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology*. 2003; 124:993–1000. [PubMed: 12671896]
33. Berrebi D, Maudinas R, Hugot JP, et al. Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut*. 2003; 52:840–846. [PubMed: 12740340]
34. Rosenstiel P, Fantini M, Brautigam K, et al. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology*. 2003; 124:1001–1009. [PubMed: 12671897]
35. van Heel DA, Ghosh S, Butler M, et al. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet*. 2005; 365:1794–1796. [PubMed: 15910952]
36. Cho JH, Abraham C. Inflammatory bowel disease genetics: Nod2. *Annu Rev Med*. 2007; 58:401–416. [PubMed: 16987083]
37. Inohara N, Ogura Y, Fontalba A, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. *J Biol Chem*. 2003; 278:5509–5512. [PubMed: 12514169]
38. Birmingham CL, Smith AC, Bakowski MA, et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem*. 2006; 281:11374–11383. [PubMed: 16495224]
39. Mukherjee K, Parashuraman S, Krishnamurthy G, et al. Diverting intracellular trafficking of Salmonella to the lysosome through activation of the late endocytic Rab7 by intracellular delivery of muramyl dipeptide. *J Cell Sci*. 2002; 115:3693–3701. [PubMed: 12186955]
40. Phillips NC, Chedid L. Anti-infectious activity of liposomal muramyl dipeptides in immunodeficient CBA/N mice. *Infect Immun*. 1987; 55:1426–1430. [PubMed: 3553001]
41. Traub S, von Aulock S, Hartung T, et al. MDP and other mucopeptides—direct and synergistic effects on the immune system. *J Endotoxin Res*. 2006; 12:69–85. [PubMed: 16690010]
42. Collins KD, Stark GR. Aspartate transcarbamylase. Interaction with the transition state analogue N-(phosphonacetyl)-L-aspartate. *J Biol Chem*. 1971; 246:6599–6605. [PubMed: 4943676]
43. Loh E, Kufe DW. Synergistic effects with inhibitors of de novo pyrimidine synthesis, acivicin, and N-(phosphonacetyl)-L-aspartic acid. *Cancer Res*. 1981; 41:3419–3423. [PubMed: 7260907]
44. Grem JL, King SA, O'Dwyer PJ, et al. Biochemistry and clinical activity of N-(phosphonacetyl)-L-aspartate: a review. *Cancer Res*. 1988; 48:4441–4454. [PubMed: 3293772]
45. Rozencweig M, Abele R, Piccart M, et al. N-(Phosphonacetyl)-L-aspartate (PALA): current status. *Recent Results Cancer Res*. 1980; 74:72–77. [PubMed: 7444151]
46. Inohara N, Koseki T, del Peso L, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem*. 1999; 274:14560–14567. [PubMed: 10329646]

47. Ogura Y, Inohara N, Benito A, et al. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem*. 2001; 276:4812–4818. [PubMed: 11087742]
48. Poyet JL, Srinivasula SM, Tnani M, et al. Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem*. 2001; 276:28309–28313. [PubMed: 11390368]
49. Shih DQ, Targan SR. Insights into IBD Pathogenesis. *Curr Gastroenterol Rep*. 2009; 11:473–480. [PubMed: 19903423]
50. Franke A, McGovern DP, Barrett JC, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet*. 2010; 42:1118–1125. [PubMed: 21102463]
51. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med*. 2010; 16:90–97. [PubMed: 19966812]
52. Travassos LH, Carneiro LA, Ramjeet M, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*. 2010; 11:55–62. [PubMed: 19898471]
53. Seidelin JB, Broom OJ, Olsen J, et al. Evidence for impaired CARD15 signalling in Crohn's disease without disease linked variants. *PLoS One*. 2009; 4:e7794. [PubMed: 19907652]

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 μ g/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.

Figure 2.

Intestinal CAD expression is altered in CD. (A–F) Immunohistochemistry performed with either (A–C) CAD antibody or (D) rabbit immunoglobulin G on colonic sections from (Control; A and D) individuals without inflammatory bowel disease, (B) CD, or (C) UC. (E) Quantification of the average CAD intensity per mucosal cell, >4 independent fields per individual quantified (control, n = 5; CD, n = 5; UC = 4). (F) Quantification of the average CAD intensity per epithelial cell as analyzed in E. Averages $\pm$ SD are shown.

Figure 3.

CAD is a negative regulator of NOD2 signaling. (A) NF- κ B reporter assay in NOD2-expressing HEK293T cells cotransfected with indicated amounts of CAD expression plasmid and MDP stimulated (10 ng/mL for 18 hours). Luciferase values normalized to β -galactosidase transfection control values (nLuc). Averages $\pm$ SD are shown. (B) NF- κ B reporter assay in NOD2-expressing HEK293T cells cotransfected with control or CAD short hairpin RNA plasmids, MDP stimulated, and assayed as in A. Immunoblot showing RNAi-mediated knockdown of Flag-CAD expression in HEK293T cells (*inset*). (C) MAPK p38 reporter assay in NOD2-expressing HEK293T cells transfected with vector or CAD expression plasmids (100 ng), stimulated with MDP (100 ng/mL for 18 hours), and analyzed as in A. (D) Immunoblots of CAD expression levels in lysates from HCT116 cells grown to different densities (20 μ g/lane; *top panel*) or immunoblots of MDP-stimulated (1 μ g/mL) NF- κ B p65 phosphorylation (p-p65; *middle panels*) or p38 phosphorylation (p-p38; *bottom panels*) in HCT116 cells grown to indicated density. (E) NF- κ B reporter assay performed as in A in HEK293T cells expressing either NOD2 or NOD1. Cells were treated with either MDP (10 ng/mL) or the NOD1 ligand KF1B (100 ng/mL) for 18 hours. (F) NF- κ B reporter assay performed as in A with cells treated with either MDP (10 ng/mL) or tumor necrosis factor α (0.1 ng/mL) for 18 hours. ** $P < .01$, *** $P < .001$.

Figure 4.

CAD modulates *Salmonella* killing in a NOD2-dependent manner. (A) Gentamicin protection assay in HCT116 cells transfected with vector or CAD expression plasmid and MDP stimulated (10 μ g/mL) during infection. Averages $\pm$ SD are shown. (B) Same as in A except cells were transfected with control or CAD short hairpin RNA plasmids 48 hours before infection. (C) Assay performed as in B except HCT116 cells were also transfected with either vector or NOD2 D291N expression construct (NOD2 DN). ** P < .01, *** P < .001.

Figure 5. Specific regions of NOD2 and CAD mediate interaction and inhibition of NOD2. (A) Lysates from HEK293T cells transfected with Flag-CAD and HA-NOD2 deletion constructs were immunoprecipitated with Flag antibody followed by immunoblot. (B) Lysates from HEK293T cells transfected with HA-Nod2 and Flag-CAD constructs and MDP stimulated (100 ng/mL for 30 minutes) were immunoprecipitated with HA antibody followed by immunoblot. (C) NF-κB reporter assay in NOD2-expressing HEK293T cells cotransfected with indicated amounts of expression plasmids and MDP stimulated (10 ng/mL for 18 hours). Luciferase values were normalized to β-galactosidase transfection control values (nLuc), and averages±SD are shown. (D) MAPK p38 reporter assay in NOD2-expressing

HEK293T cells cotransfected with indicated expression plasmids (100 ng) and MDP stimulated (100 ng/mL for 18 hours). Reporter activity assessed as in *C. (E)* Gentamicin protection assay in HCT116 cells transfected with indicated expression plasmids and MDP stimulated (10 µg/mL) during infection. Averages ±SD are shown. ** $P < .01$, *** $P < .001$.

Figure 6.

Pharmacologic inhibitors of CAD increase NOD2 function. (A) Steps of de novo pyrimidine synthesis and sites of inhibitor action. (B) Gentamicin protection assay in HCT116 cells pretreated with acivicin (10 μ mol/L for 1 hour) or PALA (250 μ mol/L for 24 hours). (C) Gentamicin protection assays in HCT116 cells transfected with either control or NOD2 short hairpin RNA plasmids 48 hours before infection and PALA pretreated (250 μ mol/L) 1 hour before infection. Averages $\pm$ SD are shown. ** P < .01, *** P < .001. (D) Immunoblot of NF- κ B p65 phosphorylation (p-p65) in HCT116 cells pretreated with PALA (250 μ mol/L for 1 hour) and MDP stimulated (10 μ g/mL). (E) Immunoblot of p38 phosphorylation (p-p38) in HCT116 cells treated as in D.

Figure 7.

Pharmacologic CAD inhibitors increase CD-associated NOD2 variant function. (A) Lysates of HEK293T cells transfected with Flag-CAD and HA-tagged NOD2 constructs were immunoprecipitated with Flag antibody followed by immunoblot. (B) NF-κB reporter assay in HEK293T cells cotransfected with control or CAD shRNA plasmids and NOD2 expression constructs. Cells were MDP stimulated (10 ng/mL for 18 hours) and luciferase values normalized to β-galactosidase transfection control values (nLuc). Averages ±SD are shown. (C) Bacterial killing of NOD2 risk variants in HEK293T cells by gentamicin protection assay in the presence or absence of MDP (10 μg/mL). (D) Gentamicin protection assay in HEK293T cells cotransfected with control or CAD short hairpin RNA plasmids and

NOD2 expression constructs. (E) Gentamicin protection assay in HEK293T cells transfected with NOD2 constructs pretreated with PALA (250 $\mu\text{mol/L}$ for 1 hour) before infection. Averages $\pm$ SD are shown. * $P < .05$, ** $P < .01$, *** $P < .001$.