Chronic Nod2 Stimulation Potentiates Activating Transcription Factor 3 and Paradoxical Superinduction of Epithelial Proinflammatory Chemokines by Mucoactive Ribotoxic Stressors via RNA-Binding Protein Human Antigen R

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Chronic exposure to gut bacteria and bacterial products including Nod2 ligands triggers homeostatic regulation in response to various mucosal insults. Activating transcription factor 3 (ATF3) is a negative regulator of proinflammatory cytokines via bacterial pattern recognition. On the assumption that ATF3 can be a critical modulator of epithelial inflammation, chronic stimulation of Nod2 was assessed for its effects on ATF3 and proinflammatory signals in response to mucosal ribotoxic insult, which is a critical etiological factor of human intestinal inflammatory disease. Muramyl dipeptide, the minimal moiety of bacterial peptidoglycan, is the Nod2 ligand, and pre-exposure to it enhanced ATF3 expression in ribotoxic stress–exposed human enterocytes. In terms of gene regulation, Nod2 preactivation potentiated ATF3 induction by enhancing stability of the ATF3 transcript, which was particularly linked to the regulation of the 3′-untranslated region of the human ATF3 gene. Moreover, chronic stimulation of Nod2 enhanced both the basal and the ribotoxic stress–stimulated cytoplasmic translocation of the HuR protein, which bound to and stabilized ATF3 messenger RNA (mRNA). Functionally, chronic stimulation of Nod2 also led to superinduction of proinflammatory chemokine genes by the mucoactive ribotoxic stress. However, the chemokine superinduction was not affected by ATF3 gene regulation although Nod2-triggered ATF3 had suppressive effects on the proinflammatory nuclear factor kappa B (NF-κB) signal. This paradoxical superinduction of chemokines was also mediated by enhanced mRNA stabilization by HuR protein in spite of ATF3-mediated suppression of NF-κB signal in human intestinal epithelial cells.

Key Words: Nod2; ATF3; chemokines; ribotoxic stress; intestinal epithelial cells.

Nod2 is a member of the nucleotide-binding domain and leucine-rich repeat containing family. Nod2 is particularly responsive to the muramyl dipeptide (MDP), N-acetylmuramyl-L-alanyl-D-isoglutamine which is a minimal peptidoglycan moiety of both Gram-positive and Gram-negative bacteria. Activation of Nod2 signals initiates a wide spectrum of innate recognition. Acute Nod2 stimulation by MDP specifically activates proinflammatory nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase cascades, which mediates defensive proinflammatory cytokine production. However, to maintain gut homeostasis, the intestinal epithelium needs to regulate detrimental cytokine production in response to chronic exposure to bacteria and bacterial products, including Nod2 ligands. Constant Nod2 activation controls overstimulation by gut microflora by triggering downregulatory machinery. Nod2 dysregulation results in an excessive intestinal inflammatory response; in particular, mutations in the Nod2 gene are among the strongest genetic risk factors in the pathogenesis of ileal Crohn’s disease (Cummings et al., 2010; Zhemakova et al., 2008). Studies have also shown that chronic Nod2 stimulation uses homotolerance or heterotolerance patterns to downregulate proinflammatory cytokines (Hedl and Abraham, 2011; Hedl et al., 2007). Here, the questions that emerge from this study begin with the hypothesis that Nod2-mediated downregulatory control of proinflammatory responses to various mucosal insults is required for intestinal homeostasis.

Stress responses by ribosome-inactivating (ribotoxic) agents that cause mucosal insults are etiological factors of epithelial inflammatory diseases and have been investigated in various experimental models (Maresca and Fantini, 2010; Thorpe et al., 2001; Yoder et al., 2007). Specific ribosome-directed xenobiotics, such as anisomycin, ultraviolet radiation, ricin, and a variety of sesquiterpenoid trichothece fungal metabolites, can damage the functionality of 28S ribosomal RNA during gene translation. This interference leads to a ribotoxic stress response that stimulates intracellular sentinel signaling pathways. This process results in the expression of genes
important for cellular homeostasis as well as genes integral to a variety of pathogenic processes involved in cell survival modulation, proliferation, and stress response (Laskin et al., 2002; Shifrin and Anderson, 1999). Several epidemiological studies suggest links between ribotoxic intoxication and human mucosal epithelial illness, including intestinal inflammatory diseases (Bhat et al., 1989; Li et al., 2002; Luo et al., 1990). Chemical ribotoxic stress alters intestinal mucosal integrity by interfering with transepithelial resistance, epithelial differentiation, and nutrient absorption, which is associated with anorexia and weight loss (Bouhet and Oswald, 2005; Calvert et al., 2005; Maresca et al., 2002; Sergent et al., 2006). Moreover, ribotoxic agents trigger intestinal inflammation as well as systemic inflammation and likely play a role in the agent’s capacity to evoe production of proinflammatory mediators in epithelial cells and other immune-related cells (Moon et al., 2008; Park et al., 2010; Zhou et al., 1999, 2005).

Activating transcription factor 3 (ATF3) is a member of the ATF/cyclic adenosine monophosphate (AMP) response element–binding family; it contains a basic region/leucine zipper DNA-binding motif and binds to the cyclic AMP response element consensus sequence (Liang et al., 1996). In most cases, the ATF3 protein is induced by various external stress signals, such as ischemic injury, mutagens, carcinogens, mitogenic cytokines, ribotoxic stress, or endoplasmic reticulum stress from abnormal protein processing (Jiang et al., 2004; Yang et al., 2009; Yin et al., 2008). ATF3 is also induced by toll-like receptor (TLR) ligands, which are included in the negative feedback loop that modulates pattern recognition receptor–stimulated inflammatory response (Gilchrist et al., 2006; Suganami et al., 2009; Whitmore et al., 2007). Particularly, ATF3 regulates gene expression of proinflammatory cytokines, such as macrophage inflammatory protein 1 beta (MIP1-β), interleukin (IL)-6, and IL-12. Recent studies have shown that ATF3 mediates epigenetic regulation of proinflammatory transcription factor NF-κB (Gilchrist et al., 2006; Li et al., 2010), which suggests that ATF3 can potentially function as a therapeutic target of regulation and tolerance in chronic inflammatory disease, including human inflammatory bowel disease.

On the assumption that ATF3 is a potent regulator of intestinal epithelial inflammation, chronic stimulation of nod2 was assessed for its effects on ATF3 and proinflammatory chemokine in response to mucosal ribotoxic insult, which is a critical factor inducing intestinal inflammatory disorder. Although chronic stimulation of nod2 can attenuate proinflammatory NF-κB signals by enhancing ATF3, mucosal ribotoxic stress–triggered proinflammatory chemokines were paradoxically enhanced by nod2 pre-exposure, which can initiate epithelial inflammatory responses in NF-κB-independent way. Mechanistically, elevated gene expression of ATF3 and chemokines was posttranscriptionally modulated by RNA-binding protein human antigen R (HuR).

**MATERIALS AND METHODS**

**Cell culture conditions and reagents.** The human epithelial cell line HCT-8, which is obtained from human embryonic jejunum and ileum, was purchased from American Type Culture Collection (Rockville, MD). The HCT-8 cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma Chemical Company, St Louis, MO), 50 U/ml penicillin (Sigma Chemical Company), and 50 μg/ml streptomycin (Sigma Chemical Company) in a 5% CO₂ humidified incubator at 37°C. The cell number was obtained by trypsin blue (Sigma Chemical Company) dye exclusion and counting in a hemocytometer. Deoxyvinalevalon (DON) was isolated with 97.6 ± 2.4% purity from Fusarium graminearum (Sigma Chemical Company). MDP and all other chemicals were purchased from Sigma Chemical Company. During all the experiments, the cells were incubated in the complete media in subconfluent state. In detail, after pretreatment with vehicle or MD, the incubated media was replaced with new treatment media containing vehicle or ribotoxins.

**Western immunoblot analysis.** Protein expression levels were compared by Western immunoblot analysis using rabbit polyclonal antibody against human actin, rabbit polyclonal anti-IκB, rabbit polyclonal anti-C/EBPβ, mouse monoclonal anti-GADD153, and mouse monoclonal anti-hnRNP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed in ice-cold phosphate buffer, lysed in boiling lysis buffer (1% [wt/vol] SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4), and sonicated for 5 s. The protein-containing lysates were quantified using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). A 50 μg of protein sample was separated by Bio-Rad mini-gel electrophoresis. The proteins were transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ); the blots were blocked for 1 h with 5% skin milk in Tris-buffered saline plus Tween 0.1% (TBST), and probed with each antibody for an additional 2 h at room temperature or overnight at 4°C. After three washes with TBST, the blots were incubated with horseradish-conjugated secondary antibody for 1 h and washed three more times with TBST. The proteins were detected by enhanced chemiluminescence substrate (Amersham Pharmacia Biotech, Piscataway, NJ).

**Conventional and real-time reverse transcription-PCR.** Total RNA was extracted using TRizol (Invitrogen) according to the manufacturer instructions. A 100 ng RNA quantity from each sample was transcribed to complementary DNA (cDNA) by using Prime RT premix (GenetBio, Nonsan, South Korea). The amplification step was performed using TaKaRa RS EX Taq DNA Polymerase (Takara Bio Inc., Shiga, Japan) in a MyCycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) using the following parameters: denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 59°C for 10 s, and elongation at 72°C for 45 s. Each PCR product aliquot was subjected to 1.2% (wt/vol) agarose gel electrophoresis and visualized by ethidium bromide staining. The 5’ forward and 3’ reverse complement PCR primers for amplification of each gene were as follows: human IL-8 (5’-ATGACTTCCAAAGTCGGCTGGCAG-3’ and 5’-TCTCGAGCCTCCTTCAAAACTCCTT-3’), human CXCL1 (5’-CTGCTTCCGTGCTC-CTGTGAC-3’ and 5’-TGAGTGTCACGTGCTGAGA-3’), mouse ATF3 (5’-CTCTGGGTCACGTGTGTG-3’ and 5’-AGGCCATCCCTGTCCTCC-TT-3’), human HuR (5’-TGAGCATCGGGAGAGGCGAAT-3’ and 5’-CCCCTTCTGGCAGCAGCCTGTA-3’), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5’-TCAAGATGTGTTGTGTATTT-3’ and 5’-CTGGTGGATGCTATCCCTCC-3’). In real-time PCR, the FAM (6-carboxyfluorescein) was used as fluorescent reporter dye and conjugated to 5’-ends of probes to detect amplified cDNA in iCycler Thermal Cycler (Bio-Rad) using the following parameters: denaturation at 94°C for 2 min and 40 cycles of reactions of denaturation at 98°C for 10 s, annealing at 59°C for 30 s, and elongation at 72°C for 45 s. Each treatment includes three replicates to ensure statistical significance and each independent experimental set was repeated two or three times. The relative quantification of gene expression was performed using the comparative Ct method. The Ct value is defined as the point where a statistically significant increase in the fluorescence has occurred. The number...
of PCR cycles (Ct) required for the FAM intensities to exceed a threshold just above background was calculated for the test and reference reactions. In all experiments, GAPDH was used as the endogenous control. Results were analyzed in a relative quantitation study with the vehicle treated. For the messenger RNA (mRNA) stability study, the cells were pretreated with vehicle or 10 μg/ml MDP for 48 h and exposed to vehicle or ribotoxins for 1 h to induce maximum mRNA level of ATF3, IL-8, or CXCL1. After stopping the transcription using 5 μM actinomycin D, the remaining amount of each mRNA was measured by RT-PCR.

Construction of plasmids. The whole coding region of ATF3 including TATAA region was generated by reverse transcription-PCR (RT-PCR) using mRNA from HCT-8 cells. The resulting 721 bp construct was cloned using TopCloner TA kit (Enzymonics, Daejeon, Korea) followed by excision at the HindIII/Nool sites, then transferred in the sense and antisense orientations into the expression plasmid pCDNA3.1Neo(+) (Invitrogen) using T4 DNA ligase (NEB, Beverly, MA), and then confirmed by DNA sequencing. The 3′-untranslated region (UTR) of the human ATF3 gene (+647/+1811) was cloned into the pGL3 control vector at the Xba I site. Cytomegalovirus (CMV) promoter-driven small hairpin interference RNA (shRNA) was constructed by inserting shRNA into pSilencer 4.1-CMV-neo vector (Ambion, Austin, TX). The control and HuR shRNA-containing vectors were constructed. HuR shRNA targeted the sequence 5′-GTCGAAAGGTTTGCTTCTT-3′.

Transfection. Cells were transfected with mixture of plasmids using Lipofectamine 2000 (Invitrogen) or Carrigene reagent (Karinove Life Sciences, Oceanside, CA) according to the manufacturer’s protocol. All transfection efficiency was maintained at around 50–60%, which was confirmed with pMX-enhanced green fluorescent protein (GFP) vector. To create stable cell lines, cells were transfected using Lipofectamine 2000 reagent. After 48 h, cells were subjected to selection for stable integrants by exposure to 700 μg/ml G418 (Invitrogen) in complete medium containing 10% FBS. Selection was continued until monolayer colonies were formed. The transfected cells were then maintained in medium supplemented with 10% FBS and 350 μg/ml G418.

Confocal microscopy. Cells were incubated in the bottom of a glass culture dish. After treatment with DON or vehicle dimethyl sulfoxide (DMSO), cells were fixed in 4% paraformaldehyde diluted in PBS. The fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min. After a 2-h block in 3% bovine serum albumin (BSA) in PBS, the cells were incubated with a 1:200 dilution of mouse polyclonal anti-HuR antibody (Santa Cruz Biotechnology) in buffer (3% BSA in PBS) at room temperature for 90 min and washed in PBS. Then, the cells were incubated with Alexa Fluor 546 goat anti-mouse IgG (H+L) for 90 min at room temperature and washed in PBS. The cells were subsequently stained with 100 ng/ml 4′-6-diamidino-2-phenylindole (DAPI, absorbance at 405 nm) in PBS for 30 min. Confocal images were obtained with an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan) using single-line excitation (546 nm) or multitrack sequential excitation (546 and 633 nm), and images were acquired and processed using FV10-ASW software.

RNA immunoprecipitation. Immunoprecipitation of protein-RNA complexes was performed using a modified protocol for chromatin immunoprecipitation (Pan et al., 2005). Briefly, HCT-8 cells were seeded at 2.5 × 10^6/100 mm dish in complete RPMI and grown for 24 h. The HCT-8 cells were then treated with vehicle control or 10 μg/ml MDP for 48 h and then exposed to the vehicle or 500 ng/ml DON (ribotoxin) for 1 h. After DON or vehicle DMSO treatment, protein and RNA were cross-linked with 1% formaldehyde. The cytoplasmic extract was incubated at 4°C overnight with 5 μg of either goat anti-mouse IgG (non-specific control) or anti-HuR antibody. The antibody-bound complex was precipitated with protein G-sepharose beads, which were then sequentially washed for 5 min each in low salt, high salt, LiCl, and TE buffers. The protein-RNA complex was eluted from the protein A-sepharose beads with 250 μl of elution buffer at 37°C for 15 min. The RNA in the immunoprecipitated complex was released by reversing the cross-linkage at 65°C by incubating for 4–5 h in 200mM NaCl and 20 μg of proteinase K. RNA was then extracted with TRIzol reagent and subjected to RT-PCR.

Statistical analysis. Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). For comparative analysis of two groups of data, Student’s t-test was used. For unpaired matched comparative analysis of multiple groups, data were subjected to ANOVA. Data that did not meet normality assumptions were subjected to Kruskal-Wallace ANOVA on ranks and then pairwise comparisons were made by the Student-Newman-Keuls (SNK) method.

RESULTS

Chronic Nod2 Stimulation Potentiates ATF3 Expression in Response to Chemical Ribosome-Inactivating Stress

MDP is the minimal moiety of bacterial peptidoglycan and the ligand for Nod2. Chronic activation of Nod2 triggers endotoxin tolerance in the mucosal immune system (Hedl et al., 2007). Based on the assumption that ATF3 plays a critical role in regulating ribotoxic stress, we investigated whether pre-exposure to the Nod2 ligand may alter ATF3 expression in ribotoxin-exposed human enterocytes. Chronic Nod2 preactivation enhanced the levels of ribotoxic stress–induced ATF3 expression (Fig. 1). Among the ribotoxic stress agents, DON has been widely detected as a ribotoxic food contaminant in cereal and various other products commonly found in the human diet (Jackson and Bullerman, 1999; Rotter et al., 1996). Particularly, natural occurrence of DON together with 15-acetylated precursor can be dominantly observed in North and South America. Depending on the ribotoxic chemicals, intestinal cells showed early or delayed ATF3 expression in response to ribotoxic stress, all of which were enhanced by chronic Nod2 preactivation. However, chronic Nod2 stimulation itself did not lead to ATF3 induction. This study focuses on posttranscriptional ATF3 regulation because transcriptional activity was only marginally altered by ribotoxic stress according to the previous report (Yang et al., 2009). Upon measurement of the ATF3 mRNA half-life, chronic stimulation of Nod2 was found to enhance the stability of the ATF3 transcript (Fig. 2A). In particular, the ribotoxin-induced ATF3 transcript was more stabilized by MDP pre-exposure than by vehicle pretreatment, which indicates a critical role for Nod2 in ATF3 stabilization. To address 3′-UTR involvement in the stabilization of ATF3 mRNA, we constructed constitutively expressing luciferase reporter plasmids tagged with the UTR of human ATF3 gene. Treatment with ribotoxin-enhanced reporter production was further enhanced by Nod2 preactivation (Fig. 2B), thereby confirming the data from Figure 2A.

Chronic Nod2 Stimulation Stabilizes Ribotoxin-Induced ATF3 mRNA via HuR Protein

Although many RNA-binding proteins regulate mRNA stability, most of these proteins affect the target mRNA stability, but there are also several known positive regulators including HuR, human antigen D, polyA-binding protein, Wig-1, and adenosine- and uridine-binding factor (Pascale et al., 2004; Rajagopalan and Malter, 1994; Vilborg et al., 2009;
Among these positive regulators, our previous study suggested the positive regulation of cytokine mRNA stability by HuR protein in response to the ribotoxic deoxynivalenol (Choi et al., 2009). In particular, HuR protein is the most extensively studied modulator. It binds to the mRNA 3'-UTR with AU-rich elements and stabilizes early-responsive genes, including growth factors, oncoproteins, and transcription factors (Chen and Shyu, 1995; Jacobson and Peltz, 1996; Sadot et al., 1995). First, the cellular localization of HuR protein was assessed in response to Nod2 activation. Because there was no change in total HuR (data not shown), the localization of HuR protein across the nuclear membrane was observed (Figs. 3A and B). Nod2 preactivation enhanced both basal and ribotoxin-stimulated cytoplasmic translocation of cytoplasmic HuR protein, which suggests that MDP and ribotoxin have additive effects on causing HuR translocation from the nucleus to the cytoplasm. Based on this observation, we hypothesized that HuR may be involved in the stabilization of ATF3 mRNA by ribotoxins or MDP. First, to assess the direct binding of the HuR protein to the ATF3 transcript, an RNA immunoprecipitation assay was performed. Nod2 preactivation was found to enhance the binding of HuR to the ATF3 transcript in both control and ribotoxin-treated cells (Fig. 4A). Moreover, the direct effects of HuR suppression on ATF3 upregulation were assessed using HuR shRNA. Although ribotoxin-induced ATF3 was not suppressed by reduced HuR expression, Nod2-enhanced ATF3 was strongly decreased by HuR shRNA (Fig. 4B), thus suggesting critical involvement of HuR protein in Nod2-enhanced ATF3 production. The data supports the enhanced stabilization of the ATF3 transcript (Fig. 2) and enhanced cytosolic translocation of HuR caused by chronic Nod2 stimulation (Figs. 3A and B). In summary, chronic stimulation of Nod2 enhances cytosolic translocation of the HuR protein, which is positively associated with the superinduction of ATF3 via mRNA stabilization in response to ribotoxic stress in human intestinal epithelial cells.
ATF3 is Negatively Associated With Proinflammatory p65 Phosphorylation in Human Intestinal Cells

Nod2-mediated superinduction of the ATF3 gene in the presence of ribotoxic stress was examined for its link to mucosal inflammatory regulation. Because ATF3 has been reported to play regulatory roles in proinflammatory cytokine production (Gilchrist et al., 2008, 2006; Li et al., 2010), it was hypothesized that chronic stimulation of Nod2 and subsequent ATF3 superinduction would suppress the proinflammatory chemokine production in response to the ribotoxic stress. However, in contrast with the expectation, chronic Nod2 stimulation enhanced the gene expression of chemokines including interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Fig. 5). Moreover, inhibition of ATF3 action using antisense ATF3 expression plasmid did not significantly affect the IL-8 production (Fig. 6A), indicating the non-involvement of ATF3 in cytokine superinduction. Additionally, chronic Nod2 stimulation enhanced the expression of chemokines including interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Fig. 5). Moreover, inhibition of ATF3 action using antisense ATF3 expression plasmid did not significantly affect the IL-8 production (Fig. 6A), indicating the non-involvement of ATF3 in cytokine superinduction. Furthermore, chronic Nod2 stimulation enhanced the expression of chemokines including interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Fig. 5). Moreover, inhibition of ATF3 action using antisense ATF3 expression plasmid did not significantly affect the IL-8 production (Fig. 6A), indicating the non-involvement of ATF3 in cytokine superinduction. Additionally, chronic Nod2 stimulation enhanced the expression of chemokines including interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Fig. 5). Moreover, inhibition of ATF3 action using antisense ATF3 expression plasmid did not significantly affect the IL-8 production (Fig. 6A), indicating the non-involvement of ATF3 in cytokine superinduction.

Enhanced Cytolic HuR by Chronic Nod2 Stimulation Contributes to Chemokine Superinduction by Ribotoxic Stress

To explain the chemokine superinduction in the ATF3- or NF-κB-independent manner, it was assumed that posttranscriptional regulation can contribute to the enhanced production of chemokines. Chronic stimulation of Nod2 extended the half-life of chemokine mRNA in response to the ribotoxic stress (Fig. 7A). Among positive regulators of mRNA stability, the cytosolic HuR protein was involved in mRNA stabilization of ATF3 (Figs. 3 and 4). Nod2 preactivation enhanced ribotoxin-triggered cytosolic translocation of HuR protein, which was associated with ATF3 superinduction. In a similar pattern, HuR was assessed for its involvement in chemokine superinduction. When HuR expression was interfered using HuR shRNA, chemokine superinduction was significantly attenuated, indicating the positive modulation of chemokine genes by HuR protein (Figs. 7B and C). Superinduction of IL-8 and CXCL1 by the ribotoxic stress was thus due to the mRNA-stabilizing HuR protein.
Intestinal epithelia are confronted with bacteria and bacterial products, including peptidoglycan and Nod2 ligands. Unlike circulating immune-related cells, intestinal epithelial cells are hyporesponsive to mucosal bacteria due to the development of regulatory machinery learned from exposure to bacteria-associated molecular patterns during gut symbiosis. Chronic stimulation of Nod2 by commensal bacteria or their Nod2 ligands attenuates the proinflammatory cytokine production upon Nod2 or TLR4 restimulation (Hedd et al., 2007). To address potent underlying mechanism of Nod2-mediated tolerance, the present study investigated ATF3 as a mucoregulatory molecule in response to epithelial ribotoxic stress, which is an etiological factor of human inflammatory bowel disease. Chronic Nod2-mediated hyporesponsiveness or tolerance means decreased proinflammatory responses to the proinflammatory triggers including bacterial products and cytokines. Nod2 pre-exposure generally desensitize activation of convergent proinflammatory transcription factor NF-κB in response to lipopolysaccharide (LPS), peptidoglycan, and cytokines. However, the mucosal ribotoxic stress can overcome such desensitization because ribotoxic stress use the NF-κB-independent pathways such as early growth response gene 1 product or posttranscriptional regulation (Choi et al., 2009; Moon et al., 2007). In the present study, Nod2 pre-exposure could not desensitize production of proinflammatory chemokines induced by ribotoxic stress because these

![Image](https://academic.oup.com/toxsci/article-abstract/125/1/116/1669219)
inductions are independent of NF-κB pathway. Nod2 pre-exposure even enhance ribotoxin-induced chemokine production by promoting more cytosolic translocation of HuR protein and its subsequent stabilizing binding to chemokine transcripts. Ribotoxic stress–induced chemokines can initiate proinflammatory gene expression in intestinal inflammatory diseases. In response to the chronic bacterial Nod2 stimulation, anti-inflammatory regulator ATF3 was potentiated in response to the mucoactive ribotoxic stress, but proinflammatory chemokines were also superinduced. However, Nod2-enhanced ATF3 could not contribute to the chemokine induction by ribotoxic stress although ATF3 had regulatory effects on NF-κB activation. Paradoxically, Nod2-associated superinduction of chemokines by ribotoxins was mediated by enhanced mRNA stabilization by HuR protein in spite of retarded NF-κB signal (Fig. 8). Increased ATF3 protein was also posttranscriptionally mediated by elevated cytosolic translocation of HuR protein and the stabilizing binding of HuR to the ATF3 transcript. Moreover, Nod2-enhanced ATF3 was negatively associated with the activation of NF-κB in human intestinal epithelial cells. In other reports, ATF3 has been shown to repress proinflammatory cytokines, such as IL-6, IL-12, and macrophage inflammatory protein-1β (MIP-1β) via binding to the ATF/CRE-binding site on the promoter of each gene (Gilchrist et al., 2006; Li et al., 2010; Whitmore et al., 2007). In LPS-exposed mice, ATF3-knockout animals show a higher titer of proinflammatory cytokines than that shown by wild-type mice, which implicates regulatory effects of ATF3 on the inflammatory response. Mechanistically, ATF3 as a transcriptional repressor reduces gene transcription of pathogen-associated molecular pattern-induced cytokines by interfering with NF-κB-mediated transcriptional activation (Suganami et al., 2009). ATF3-mediated regulation can also be explained at the epigenetic level; ATF3 recruits histone deacetylase (HDAC) 1 to the ATF/NF-κB sites of the cytokine gene promoters, which leads to modification of histone proteins, condensation of

**FIG. 5.** Superinduction of proinflammatory chemokines in response to the ribotoxic insults. (A and B) HCT-8 cells were pretreated with a vehicle control or 10 μg/ml MDP for 48 h and then exposed to the vehicle or 500 ng/ml DON (A) and anisomycin (B) for 1 h. Each chemokine mRNA was measured using real-time RT-PCR. The different letter over each bar with the SD represents significant differences between two groups by unpaired matched comparisons (p < 0.05). Results are representative of three independent experiments.

**FIG. 6.** Effects of ATF3 suppression on chemokine and proinflammatory Figure 2. NF-κB activation in human intestinal epithelial cells. (A) Control and antisense ATF3-expressing HCT-8 cells were pretreated with a vehicle control or 10 μg/ml MDP for 48 h and then exposed to the vehicle or 500 ng/ml ribotoxin DON for 12 h. Secreted IL-8 was measured using ELISA method. The different letter over each bar with the SD represents significant differences between two groups by unpaired matched comparisons (p < 0.05). Results are representative of two independent experiments. (B) Control and antisense ATF3-expressing HCT-8 cells were pretreated with a vehicle control or 10 μg/ml MDP for 48 h and then exposed to the vehicle or ribotoxins (500 ng/ml DON) for indicated times. The total cellular lysate was subjected to Western blot analysis. Results are representative of two independent experiments.
chromatin structure, and suppression of target gene expression (Li et al., 2010). Thus, it is necessary to investigate the epigenetic details of ATF3-mediated regulation in response to Nod2 and ribotoxic stress in the future study.

In addition to the ATF3 regulatory response to bacterial exposure, chronic Nod2 activation triggers several other regulatory cellular signals, such as interleukin-1 receptor-associated kinase M and mammalian target of rapamycin, which also block NF-κB-linked cellular events, including proinflammatory response genes in defense against external stimuli (Hedl and Abraham, 2011; Hedl et al., 2007). Another NF-κB target is the HuR protein, which is upregulated via PI3K-AKT signaling in gastric cancer cells (Kang et al., 2008). HuR is known to function as a critical stabilizing mediator of early-responsive genes in response to diverse stress conditions (Choi et al., 2009; Kim et al., 2003; Oyesanya et al., 2008). Based on these previous reports, we hypothesized that constitutive triggering of NF-κB in Nod2-exposed cells increases the levels of HuR protein expression and enhances subsequent ATF3 stabilization. In other words, mucosal epithelia that are constitutively faced with bacterial inflammmogens frequently stimulate NF-κB signals, which can increase HuR basal expression and increased cytosolic translocation of HuR protein in response to ribotoxic stress, thereby increasing HuR binding to ATF3 transcript in the cells. Thus, enhanced ATF3 downregulates NF-κB activation as a feedback mechanism in normal intestinal mucosa, which explains why commensal bacterial endotoxin-activated NF-κB is generally transient and chronic Nod2 activation induces a regulatory response against the proinflammatory stimuli (Hedl et al., 2007).

Nod2 mediates the host recognition of bacterial peptidoglycan moiety and is a critical regulator of commensal microbiota in the intestine (Petnicki-Ocwieja et al., 2009). Moreover, chronic stimulation of Nod2 mediates mucosal tolerance to bacterial products by attenuating proinflammatory cytokine production (Hedl et al., 2007). However, chronic Nod2 preactivation led to superinduction of epithelial proinflammatory chemokines by the mucoactive ribotoxic stress. By contrast, chronic stimulation of another pattern recognition receptor TLR4 suppresses cytokine production via the delayed induction of peroxisome proliferator–activated receptor gamma (PPAR-γ), which contributes to the diminished IL-8 production in the human epithelial cells. Because chronic Nod2 stimulation also can enhance basal PPAR-γ levels, extended study is warranted to address the difference in the responses to chronic Nod2 and TLR4 stimulation. In response to chronic TLR4

**FIG. 7.** Involvement of HuR protein in chronic Nod2-triggered superinduction of chemokines in response to the ribotoxic stress. (A) HCT-8 cells were pretreated with a vehicle or with 10 μg/ml MDP and then exposed to the vehicle or 500 ng/ml DON for 1 h to reach maximum level of IL-8 or CXCL1; transcription was then determined by adding 5μM actinomycin D. The remaining ATF3 mRNA was measured. (B) Control and HuR shRNA-expressing HCT-8 cells were pretreated with a vehicle control or 10 μg/ml MDP for 48 h and then exposed to the vehicle or 500 ng/ml ribotoxin DON for 1 h. Each chemokine mRNA was measured using real-time RT-PCR. The different letter over each bar with the SD represents significant differences between two groups by unpaired matched comparisons (p < 0.05). Results are representative of two independent experiments.
Despite the positive role of ATF3 in inflamed epithelial tissues, modulation on the epithelial carcinogenesis should be addressed. Potential adverse effects of Nod2 preactivation by bacterial stimulation can be harmful during epithelial cancer progression. Moreover, our recent report describes an ATF3 association with cellular survival response to chemical ribotoxic stress. However, the enhanced survival induced by ATF3 may facilitate the passage of tumor cells through a cytotoxic environment and provide tumor growth advantage over the normal cells. Although the regulatory action of ATF3 can be beneficial to inflamed mucosa, prolonged ATF3 expression can be harmful during epithelial cancer progression. Furthermore, the oncogenic potential of ATF3 implicates the epithelial carcinogenesis should be addressed, despite the positive role of ATF3 in inflamed epithelial tissues.

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