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*J Immunol* 2013; 191:5170-5181; Prepublished online 4 October 2013; doi: 10.4049/jimmunol.1301145

http://www.jimmunol.org/content/191/10/5170

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/10/04/jimmunol.1301145.DC1

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Novel Regulatory Action of Ribosomal Inactivation on Epithelial Nod2-Linked Proinflammatory Signals in Two Convergent ATF3-Associated Pathways

Seong-Hwan Park,*† Kee Hun Do,*† Hye Jin Choi,*† Juil Kim,*† Ki-Hyung Kim,‡ Jiyeon Park,*† Chang Gyu Oh,*† and Yuseok Moon*†

In response to excessive nucleotide-binding oligomerization domain–containing protein 2 (Nod2) stimulation caused by mucosal bacterial components, gut epithelia need to activate regulatory machinery to maintain epithelial homeostasis. Activating transcription factor 3 (ATF3) is a representative regulator in the negative feedback loop that modulates TLR-associated inflammatory responses. In the current study, the regulatory effects of ribosomal stress-induced ATF3 on Nod2-stimulated proinflammatory signals were assessed. Ribosomal inactivation caused persistent ATF3 expression that in turn suppressed proinflammatory chemokine production facilitated by Nod2. Decreased chemokine production was due to attenuation of Nod2-activated NF-κB and early growth response protein 1 (EGR-1) signaling by ATF3. However, the underlying molecular mechanisms involve two convergent regulatory pathways. Although ATF3 induced by ribosomal inactivation regulated Nod2-induced EGR-1 expression epigenetically through the recruitment of histone deacetylase 1, NF-κB regulation was associated with posttranscriptional regulation by ATF3 rather than epigenetic modification. ATF3 induced by ribosomal inactivation led to the destabilization of p65 mRNA caused by nuclear entrapment of transcript-stabilizing human Ag R protein via direct interaction with ATF3. These findings demonstrate that ribosomal stress-induced ATF3 is a critical regulator in the convergent pathways between EGR-1 and NF-κB, which contributes to the suppression of Nod2-activated proinflammatory gene expression. The Journal of Immunology, 2013, 191: 5170–5181.

Human intestinal epithelium is a frontline defense against luminal commensal bacteria. Contact between the intestinal epithelium and microorganisms is facilitated through interactions between the pattern recognition receptors (PRRs) and bacterial Ags (1). In response to N-acetylMuramyl-L-alanyl-d-isoglutamine (MDP), a minimal peptidoglycan moiety of both Gram-positive and Gram-negative bacteria, nucleotide-binding oligomerization domain–containing protein 2 (Nod2) is activated and initiates a wide spectrum of innate recognition and immune responses (2, 3). Acute Nod2 stimulation by MDP specifically activates proinflammatory NF-κB and MAPK cascades that mediate the expression of proinflammatory mediators, including cytokines (4, 5). Although Nod2 usually triggers proinflammatory responses, Nod2-induced signals are also crucial for intestinal homeostasis because various Nod2 gene mutations are found in patients with Crohn’s disease, a type of inflammatory bowel disease (IBD) characterized by severe damage to intestinal tissues (6–8). Mechanistically, Nod2 increases the expression of defensin, a factor that contributes to gut protection against harmful bacteria.

To maintain gut homeostasis in the presence of luminal bacteria, the intestinal epithelia are hyporesponsive to bacterial inflammasomes via regulatory mechanisms (9). As a representative negative regulator of the PRR-linked signals, the peroxisome proliferator–activated receptor γ (PPARγ) mediates immune tolerance to bacteria-stimulated inflammation associated with colitis (10–12). Genetic ablation of PPARγ is observed in patients with ulcerative colitis, which is associated with severe chronic inflammatory outcomes (10). As another regulatory transcription factor, activating transcription factor 3 (ATF3) is induced as part of the negative feedback loop that modulates PRR-stimulated inflammatory responses (13–15). In particular, ATF3 regulates the expression of proinflammatory cytokine genes, such as ones encoding MIP1-β, IL-6, and IL-12. Not surprisingly, ATF3-deficient macrophages produce elevated levels of IL-6 and IL-12p40 cytokines in response to TLR 4 activator, a bacterial LPS (13, 15). Mechanistically, the negative regulation of transcription by ATF3 may occur indirectly via inhibition of C/EBPβ, a positive regulator of cytokine gene induction (16). In addition, recent studies have shown that ATF3 mediates epigenetic regulation of proinflammatory cytokines (13, 17). This finding suggests that ATF3 can potentially function as a therapeutic target of immune
regulation and tolerance in patients with chronic inflammatory diseases, including IBD.

On the basis of the assumption that epithelial ATF3 can promote tolerance to Nod2-associated proinflammatory activation in the intestine, specific ribosome-directed xenobiotics were used as potent ATF3 modulators and analyzed for their regulatory actions on Nod2-stimulated proinflammatory signals. Ribosome-inactivating xenobiotics such as anisomycin (ANS), UV radiation, ricin, and a variety of sesquiterpenoid trichothecene fungal metabolites can disrupt 28S rRNA during gene translation (18, 19). This disruption elicits a ribotoxic stress response that activates intracellular signaling pathways. Ribosomal inactivation results in the expression of genes, such as ATF3, that are important for cellular homeostasis and of genes essential for a variety of processes involved in cell survival, proliferation, and death, as well as inflammatory responses (20, 21). In the current study, ribosomal inactivation was used as the inducer of prolonged ATF3 expression and assessed for its usefulness in epithelial hyporesponsiveness to Nod2 stimulation, and the underlying molecular mechanism of ATF3-linked action was addressed.

Materials and Methods

Cell culture and reagents
Human epithelial cell lines, including HCT-8, HCT-116, and intestinal 407 cells, were purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO), 50 U/ml penicillin (Sigma-Aldrich), and 50 μg/ml streptomycin (Sigma-Aldrich) in a 5% CO2 humidified incubator (Sanyo, Tokyo, Japan) at 37°C. Medium for the intestinal 407 cells was additionally supplemented with 1% nonessential amino acids (Invitrogen). Moreover, we isolated the mouse enterocytes to validate the cell line–based results. Briefly, 6-wk-old male C57BL/6 mice were anesthetized and exsanguinated, and the entire small intestine was opened longitudinally and washed in 20 ml HBSS several times until luminal matter was completely removed by inverting. The intestine was then cut into 0.5-mm-long fragments and incubated in 5 ml prewarmed 0.05% trypsin for 20 min at 37°C. After incubation, the detached enterocytes were passed through the cell strainer and collected in RPMI 1640 medium. Cells were separated by centrifugation in the 25/40% discontinuous Percoll (Sigma-Aldrich) gradient at 600 × g for 10 min. Small-intestinal epithelial cells were collected from the interface between the two different Percoll gradients and resuspended in RPMI 1640 media. Cell number was determined by trypsin blue (Sigma-Aldrich) dye exclusion using a hemocytometer. Deoxyribonuclease (DON) (97.6 ± 2.4% pure) isolated from Fusarium graminearum was obtained from Sigma-Aldrich. Bay 11-7082 was purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich.

Construction of plasmids

The cDNA containing the entire coding region of ATF3, including the TATAAA region, was generated by RT-PCR using RNA from HCT-8 cells with the following primers: 5’-CTGAGTCCGTGCTC-3’ (forward) and 5’-GACAGGTTCCATTGCTC-3’ (reverse). The resulting 721-bp fragment was cloned into a TopClon TA Kit (Enzynomics, Daejeon, South Korea) at 37°C. The resulting plasmid was transformed into E. coli DH5α for PCR analysis and visualized by staining with ethidium bromide. The following 5’- and 3’-oligonucleotides were used for conventional and real-time RT-PCR: mouse IL-8 (5’-GATGACTTGCCTCGGATGGTTCCTGTTGC-3’ and 5’-TGTGCTGCTGCTGATGAG-3’), human IL-8 (5’-GATGACTTGCCTCGGATGGTTCCTGTTGC-3’ and 5’-TGTGCTGCTGCTGATGAG-3’), human EGR-1 (5’-CTGAGGTTCCATTGCTC-3’ and 5’-GACAGGTTCCATTGCTC-3’), mouse CXCL-2 (5’-GGGGCAACCCCTTGTAAGAG-3’ and 5’-GGGGCAACCCCTTGTAAGAG-3’), mouse CXCL-1 (5’-GGGGCAACCCCTTGTAAGAG-3’ and 5’-GGGGCAACCCCTTGTAAGAG-3’), human ATF3 (5’-CTGAGGTTCCATTGCTC-3’ and 5’-GACAGGTTCCATTGCTC-3’), human GAPDH (5’-TCAACGGATTTGCTGTAATTTG-3’ and 5’-CTGAGGTTCCATTGCTC-3’), mouse CCL-2 (5’-GGGGCAACCCCTTGTAAGAG-3’ and 5’-GGGGCAACCCCTTGTAAGAG-3’).
TTA GCC TTG CCT TTC A-3), mouse MCP-1 (5'-CAG ATG CAG TTA ACG CCC CA-3' and 5'-TGG AAT CCT GAA CCC ACT TC-3'), and mouse GAPDH (5'-TCA ACG GAT TTG GCC GTATT-3' and 5'-CTG TGG TCA TGA GCC CTT CC-3'). For real-time PCR, FAM was used as a fluorescent reporter dye and conjugated to the 5' ends of the probes used to detect the amplified cDNA. Real-time PCR was performed with an iCycler Thermal Cycler (Bio-Rad), using the following parameters: denaturation at 94˚C for 2 min followed by 40 cycles of denaturation at 98˚C for 10 s, annealing at 59˚C for 30 s, and elongation at 72˚C for 45 s. Each sample was tested in triplicate to ensure statistical significance. Relative quantification of gene expression was performed using the comparative Ct method. For this, the Ct value is defined as the point at which a statistically significant increase in fluorescence is observed. The number of PCR cycles (Ct) required for the FAM intensities to exceed a threshold value just above background was calculated for the test and reference reactions. In all experiments, GAPDH was used as the internal control. Results were analyzed in a relative quantitation study with the vehicle treated.

**FIGURE 1.** Effects of ATF3 on Nod2-linked proinflammatory signals in human enterocytes. (A) After HCT-8 cells transfected with ATF3-SC or an empty vector were pretreated with a ribosomal inactivator (500 ng/ml DON) for 9 h, the cells were then treated with the vehicle (PBS) or 10 µg/ml MDP for 18 h. The concentration of IL-8 secreted into the culture media was then measured using an ELISA. Blots to the left in the box show ATF3 expression in the transfected HCT-8 cells. Blots to the right in the box show ATF3 expression induced by ribosomal-inactivating DON in the presence of MDP. Total cell lysates were subjected to Western blot analysis. *A significant difference between two groups (p < 0.05). (B) HCT-8 cells pretreated with 500 ng/ml DON for 9 h, ANS for 24 h, or 15-acetyl DON (15-AcDON) for 9 h were incubated with the vehicle (PBS) or 10 µg/ml MDP for 18 h. IL-8 secreted into the culture medium was quantitated using an ELISA. *A significant difference from the group treated with MDP alone (p < 0.05). (C) HCT-8 cells transfected with an empty vector or shATF3 were pretreated with DMSO or 500 ng/ml DON for 9 h. The cells were then treated with the vehicle (PBS) or 10 µg/ml MDP for 18 h. IL-8 secreted into the culture medium was quantitated using an ELISA. Different letters over each bar with the SD represent significant differences between the two groups (p < 0.05). (D) HCT-8 cells transfected with an empty vector or shATF3 were pretreated with DMSO or 500 ng/ml ANS for 24 h. The cells were then treated with the vehicle (PBS) or 10 µg/ml MDP for 18 h. IL-8 secreted into the culture medium was quantitated using an ELISA. The figures in the box represent the relative levels of ATF3 mRNA in HCT-8 cells transfected with an empty vector or shATF3. *A significant difference between two groups (p < 0.05).
Confocal microscopy

Cells were incubated in a glass-bottom culture dish. After pretreatment with DMSO as vehicle or ribotoxins, the cells were treated with 10 μg/ml MDP. The cells were fixed with 4% paraformaldehyde (Biosesang, Sungnam, South Korea). The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. After 2-h blocking with 3% BSA in PBS, the cells were incubated at room temperature for 2 h with target Ab diluted 1:200 in a buffer (3% BSA in PBS). Next, the cells were repeatedly washed with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H + L), Alexa Fluor 546 goat anti-mouse IgG (H + L), or Texas Red (SurModics, Eden Prairie, MN) for 2 h at room temperature. The cells were repeatedly washed in PBS and subsequently stained with 100 ng/ml DAPI (absorbance at 405 nm) in PBS for 10 min. Confocal images were obtained with an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan) using single-line excitation (488 nm for anti-rabbit Abs or 546 nm or 633 nm for anti-mouse Abs) or multitrack sequential excitation (488 nm and 633 nm). Images were acquired and processed with FV10-ASW software.

Chromatin IP assay

Cells were cross-linked for 10 min in 1% formaldehyde. The reaction was stopped by the addition of glycine at a final concentration of 125 mM, and the cells were washed twice with 1× PBS. Chromatin was fragmented to a size of 1000–2000 bp by sonication for 10 s of nine times in lysis buffer [1% (w/v) SDS, 10 mM EDTA (pH 8.0), 50 mM Tris-HCl (pH 8.0), and a protease inhibitor mixture] using a Vibra-Cell sonicator (Sonics and Materials, Newtown, CT). The soluble chromatin was immunoprecipitated with 2 μg target Ab in a solution of nine parts dilution buffer [1% Triton X-100, 150 mM NaCl, 2 mM EDTA (pH 8.0), 20 mM Tris (pH 8.0), and protease inhibitor mixture] and one part lysis buffer. After rotating overnight at 4°C, protein G PLUS-agarose (30 μl; Santa Cruz Biotechnology) was added to each chromatin/Ab sample in a 100-μl volume containing a 9:1 mixture of dilution buffer and lysis buffer with 100 μg/ml salmon sperm DNA (Invitrogen). After centrifugation, each sample was washed twice in dilution buffer. The chromatin was resuspended in a dilution buffer/lysis buffer (9:1) solution and incubated at 37°C with proteinase K and RNase A (500 μg/ml per sample) for 1 h. Chromatin was purified using a MEGAquick-spin kit (iNtRON, Sungnam, South Korea). The recovered chromatin was amplified with HS Prime Taq DNA Polymerase (Genet Bio, Nonsan, South Korea). Amplification was performed with Takara HS ExTaq DNA Polymerase (Takara Bio) in a Mycycler Thermal Cycler (Bio-Rad). The following 5′ forward and 3′ reverse-complement PCR primers were used for amplification: human EGR-1 promoter (5′-CTA GGG TGC AGG ATG GAG GT-3′) and 5′-GAA CAC TGA GGC GTC AG-3′).

RNA IP

Immunoprecipitation of protein–RNA complexes was performed using a modified protocol for chromatin IP (23). Briefly, HCT-8 cells were seeded at a density of 2.5 × 10⁵ cells per 100-mm dish in complete RPMI 1640 and grown for 24 h. The cells were pretreated with 500 ng/ml DON (ribotoxin), and then incubated with 10 μg/ml MDP for 2 h. After treatment, protein and RNA were cross-linked with 1% formaldehyde for 10 min at room temperature. The cytoplasmic extract was incubated overnight at 4°C with 5 μg of either goat anti-mouse IgG (nonspecific control) or anti-HuR Ab. The Ab-bound complexes were precipitated with protein G–Sepharose beads, and then sequentially washed in low-salt, high-salt, LiCl, and TE buffers (5 min per wash). The protein–RNA complexes were eluted from the protein G–Sepharose beads with 250 μl elution buffer at 37°C for 15 min. RNA in the immunoprecipitated complexes was released by reversing the cross-linkage by incubating at 65°C for 4–5 h in 200 mM NaCl and 20 μg 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). To compare two groups of data, the Student t test was used. To
compare multiple groups, data were subjected to an ANOVA, and pairwise comparisons were made using the Student–Newman–Keuls method. Data not meeting the normality assumptions were subjected to Kruskal–Wallis ANOVA by ranks, and pairwise comparisons were then made with the Student–Newman–Keuls method.

**Results**

*Nod2-induced proinflammatory chemokines are suppressed by induced ATF3 in human enterocytes*

Intracellular accumulation of bacterial products such as MDP triggers signals that lead to the initiation of intestinal inflammatory responses via NF-κB signaling, particularly during the development of IBD (24, 25). The basic hypothesis is that ATF3 can attenuate MDP-linked proinflammatory responses in human intestinal epithelial cells. In the current study, induction of ATF3 by ribosomal inactivation or plasmid transfection suppressed IL-8 production promoted by Nod2 ligand in HCT-8 human intestinal epithelial cells (Fig. 1A). The blots shown in Fig. 1A (box) confirmed findings from our previous report demonstrating that ATF3 expression is induced by the ribosomal inactivator DON in human enterocytes (26). In the current study, the effects of ATF3 or ATF3-inducing ribosomal inactivation on chemokine expression were assessed in cultured HCT-8 intestinal epithelial cells, which are frequently used as a model of microbial infection and inflammatory diseases (27–29). Moreover, the source of HCT-8 cells, the ileocecum of the human small intestine, is one of the organs most susceptible to the biological effects of ribosomal inactivation (30, 31). In addition to DON, increased ATF3 expression promoted by other ribosome-inactivating agents, such as 15-AcDON and ANS, also suppressed MDP-induced IL-8 production in a dose-dependent manner (Fig. 1B). The direct involvement of ATF3 in the suppression of IL-8 production by ribosomal inactivation was assessed using ATF3 shRNA. Ablation of ATF3 expression lessened the suppressive effect of ribosome-inactivating agents (DON and ANS) on Nod2-activated IL-8 production (Fig. 1C, 1D). In addition, ribosomal inactivation or ATF3 overexpression downregulated MDP-induced mRNA expression of other chemokines (Fig. 2A, 2B), including MCP-1 and CXCL-1. Similar suppressive effects on chemokine expression by ribosomal inactivation were also observed in other intestinal epithelial cells, such as HCT-116 and INT-407 (Fig. 2C, 2D). Moreover, the chemokine suppression was validated in the primary mouse enterocytes as well (Supplemental Fig. 1A). Finally, the suppression of ATF3 expression using shRNA was shown to attenuate the action of ribosome-inactivating agents (DON and ANS) in human enterocytes (Fig. 3A, 3B), demonstrating the direct involvement of ATF3 in the reduction of chemokine expression by ribosomal inactivation. Taken together, these data suggest that ribosomal inactivation suppresses Nod2-induced proinflammatory chemokine expression via ATF3 in human enterocytes.

**FIGURE 3.** Effect of ATF3 on the expression of proinflammatory chemokines altered by ribosomal inactivation. (A) HCT-8 cells transfected with an empty vector or shATF3 were pretreated with DMSO or 500 ng/ml DON for 9 h and subsequently treated with the vehicle (PBS) or 10 μg/ml MDP for 2 h. The mRNA expression was measured using real-time RT-PCR. (B) HCT-8 cells transfected with an empty vector or shATF3 were pretreated with DMSO or 500 ng/ml ANS for 24 h and then incubated with the vehicle (PBS) or 10 μg/ml MDP for 2 h. The mRNA expression was measured using real-time PCR. The figures in the box represent the relative levels of ATF3 mRNA in HCT-8 cells transfected with an empty vector or shATF3. *A significant difference compared with the group treated with MDP alone (p < 0.05). ^A significant difference from the group of empty vector-transfected cells under the treatment with MDP/ribosomal inactivation (p < 0.05).
ATF3 induction suppresses proinflammatory transcription factors NF-κB and EGR-1 promoted by epithelial Nod2

Among the various proinflammatory transcription factors, NF-κB plays central roles in Nod2-activated proinflammatory responses in human enterocytes (32–35). Moreover, the expression of some NF-κB–associated proinflammatory transcription factors such as EGR-1 can be induced by a range of stimuli, including NF-κB activators (36–38). As expected, both NF-κB and EGR-1 were positively involved in chemokine induction by Nod2 activation in the current study (Fig. 4A). This finding was observed using EGR-1 shRNA and Bay 11–7082, a specific NF-κB inhibitor. Epithelial Nod2 activation also enhanced EGR-1 expression as well as p65 phosphorylation (Fig. 4B). However, pre-exposure to ATF3-inducing ribosomal inactivation using DON attenuated EGR-1 and NF-κB activation in a dose-dependent manner. In contrast to the suppression of EGR-1 and NF-κB, ATF3 expression was gradually enhanced by increasing doses of ribosome-inactivating DON, indicating that ATF3 expression may be inversely associated with that of EGR-1 and NF-κB in response to Nod2 stimulation in HCT-8 epithelial cells. Similar patterns of Nod2-activated transcription factor were also observed in cells subjected to other ribosomal insults, including ANS and 15AcDON (Fig. 4C). Moreover, these were validated in the primary mouse enterocytes (Supplemental Fig. 1B). The effects of ATF3 overexpression on
Nod2-activated EGR-1 and NF-κB were also confirmed with confocal microscopy (Fig. 4D). Nod2 activation enhanced the expression and nuclear localization of EGR-1 and p65 in human enterocytes. This result was attenuated by elevated ATF3. Ribosome-inactivating compounds that increased ATF3 expression also caused similar reductions of Nod2-activated EGR-1 and p65 in the nuclei (Fig. 4E). These findings indicated that ATF3 is negatively associated with proinflammatory transcription factors such as EGR-1 and NF-κB in response to Nod2 stimulation. Finally, the direct involvement of ATF3 in the regulation of Nod2-activated proinflammatory EGR-1 and NF-κB was assessed. Ablation of ATF3 expression with shRNA lessened the suppressive effects of ribosome-inactivating agents (DON or ANS) on proinflammatory NF-κB and EGR-1 activated by Nod2 ligand in human enterocytes (Fig. 4F, 4G). Taken together, results of these experiments demonstrated that increased ATF3 expression induced by ribosomal inactivation mediated the suppression of Nod2-activated NF-κB and EGR-1 expression, which contributed to reduced chemokine production in Nod2-activated enterocytes.

Ribosomal inactivation–induced ATF3 epigenetically regulates Nod2-induced EGR-1 expression via HDAC1

Because epigenetic modification is a well-known ATF3-associated regulatory mechanism of NF-κB activation by bacterial LPS, we assessed the effect of HDAC inhibition on ATF3-mediated regulation of Nod2-stimulated NF-κB and EGR-1 expression. Contrary to our expectations, chemical HDAC inhibition using trichostatin A did not attenuate the reduction of NF-κB by ribosomal inactivation (Fig. 5A). Instead, HDAC inhibition restored EGR-1 expression in response to Nod2 activation. Similar patterns were also observed in cells deficient in HDAC1, using HDAC1 shRNA (Fig. 5B). Therefore, ATF3-mediated epigenetic regulation appears to be more critical for EGR-1 expression than in NF-κB activated by Nod2 ligand, indicating the presence of convergent regulatory pathways for NF-κB and EGR-1 signaling.

It was next determined whether ribosomal inactivation–induced ATF3 translocates into the nuclei and can interact with the ATF/CREB element of the EGR-1 promoter. Ribosomal inactivation strongly enhanced ATF3 binding to the EGR-1 promoter; this result was partly decreased by Nod2 activation (Fig. 6A). Confocal microscopy demonstrated that ribosomal inactivation enhanced ATF3 expression and its nuclear interaction with HDAC1, and Nod2 activation itself had little effect on this interaction (Fig. 6B). Our IP assay also confirmed that ATF3 and HDAC1 interacted in cells subjected to ribosomal inactivation (Fig. 6C). In summary, these data show that ribosomal inactivation increased the recruitment of HDAC1 by ATF3. This accounted for the epigenetic regulation of EGR-1 transcription in response to Nod2 activation in the epithelial cells. In contrast to the previously known mechanism of epigenetic regulation of NF-κB by ATF3, HDAC1-mediated epigenetic regulation by ATF3 did not contribute to the inhibition of NF-κB expression by ribosomal inactivation.

Ribosomal inactivation–induced ATF3 suppresses Nod2-activated NF-κB through p65 mRNA destabilization

Because NF-κB signal was not epigenetically regulated by ribosomal inactivation, other gene regulation modes such as transcription and posttranscription were assessed. Along with reduced phosphorylation of NF-κB, total expression of p65, a central subunit of canonical NF-κB, was also decreased by ribosomal inactivation (Fig. 7A). Moreover, exogenous ATF3 overexpression subsequently suppressed both p65 expression and phosphorylation (Fig. 7B). However, because ribosomal inactivation did not affect p65 transcriptional activity (data not shown), it was therefore assumed that ribosomal inactivation may lead to alterations in p65 mRNA stability via ATF3. Measurement of p65 mRNA stability demonstrated that ribosomal inactivation significantly destabilized p65 mRNA (Fig. 7C). This stability was restored by suppressing ATF3 expression using shRNA (Fig. 7C). Moreover, the destabilization of p65 mRNA by ribosomal inactivation was also validated in the primary mouse enterocytes (Supplemental Fig. 1C).

The mechanism underlying ATF3-mediated posttranscriptional regulation was assessed in enterocytes treated with Nod2 ligand. As mediators of posttranscriptional regulation, RNA-binding proteins may play important roles in ATF3-associated mRNA destabilization. Although most RNA-binding proteins destabilize targeted mRNA, a few of these proteins, including HuR, bind to 3‘ untranslated

**FIGURE 5.** Involvement of HDAC1 in ATF3-regulated EGR-1 expression. (A) After HCT-8 cells were pretreated to 500 ng/ml DON, with or without 80 nM trichostatin A (TSA), for 9 h, the cells were treated with 10 μg/ml MDP for the indicated time. Cell lysates were subjected to Western blot analysis. (B) HCT-8 cells transfected with an empty vector or shHDAC1 were pretreated with 500 ng/ml DON for 9 h. The cells were then incubated with 10 μg/ml MDP for the indicated time. Cell lysates were subjected to Western blot analysis. The figures in the box represent the relative levels of HDAC1 mRNA in HCT-8 cells transfected with an empty vector or shHDAC1. The mRNA expression was measured using RT-PCR.
regions with AU-rich elements and stabilize the transcripts of genes, including ones encoding growth factors or proinflammatory cytokines (33, 39). In the current study, the impact of HuR on p65 mRNA stabilization was assessed. In addition to ATF3 overexpression and ATF3-inducing ribosomal inactivation, ablation of HuR expression using shRNA suppressed p65 protein expression (Fig. 8A), and the blocking of HuR expression, using its shRNA, decreased the half-life of p65 mRNA (Fig. 8B), indicating that HuR mediates p65 mRNA stabilization. In further examination of HuR function, we determined whether ATF3 can modulate the behavior of the HuR protein. Although total levels of HuR protein were only marginally altered, Nod2 activation enhanced the cytosolic translocation of HuR protein (Fig. 8C). This result was suppressed by ATF3 expression or ribosomal inactivation, indicating that ATF3 may inhibit the cytosolic release of HuR protein. Interaction between ATF3 and HuR protein in the nuclei was also assessed (Fig. 8D). Most ATF3 entered the nuclei and bound to nuclear HuR through direct interaction visualized with confocal microscopy (Fig. 8E). This interaction may limit HuR-mediated functions in the cytoplasm, including the stabilization of p65 transcript. As expected, although cytosolic p65 mRNA binding to HuR protein was enhanced by Nod2 activation, increased ATP3
expression by ribosomal inactivation reduced the levels of HuR-bound p65 mRNA (Fig. 8F). As a result, increased ATF3 levels led to a reduction of nuclear and total p65 protein levels (Fig. 8G). Taken together, results from these experiments indicated that ATF3 destabilized p65 mRNA by inhibiting HuR cytosolic translocation through direct interaction in the nuclei of human enterocytes (Fig. 9).

**Discussion**

The present study suggested new mechanisms underlying ATF3-mediated regulation of Nod2-activated proinflammatory signals in enterocytes. In particular, ATF3 overexpression or ATF3-inducing ribosomal inactivation suppressed the production of proinflammatory chemokines owing to decreased expression of NF-κB and EGR-1. ATF3 expression can also be induced by TLR
activation by bacterial infection, ischemia-reperfusion injury, or stress-induced inflammatory responses (13, 40, 41). These previous studies have indicated that ATF regulates NF-κB expression through epigenetic or transcriptional mechanisms (13, 14). Instead of epigenetic or transcriptional regulation, the current study demonstrated that Nod2-activated p65 is rather regulated by ATF3 at the posttranscriptional level. Stabilization of the p65 transcript was decreased because the RNA-binding protein HuR was sequestered in the nuclei via direct interaction with ATF3 (Fig. 9). Compared to other factors that promote ATF3 expression, ribosomal inactivation induced the expression of ATF3, which was prolonged for a relatively long time (24–48 h). Such an extended period of ATF3 expression thus triggered pathways that regulate p65 expression in response to Nod2 ligand. According to our previous study, ribosomal inactivation can enhance the transient cytosolic release of HuR protein (42). However, chronically induced ATF3 expression by ribosomal inactivation contributed to the nuclear entrapment of HuR protein via direct interaction with ATF3, which in turn caused the destabilization of p65 mRNA in the current study.

So far, only a few studies have reported on the regulation of p65 expression at the posttranscriptional level (43, 44). Although the ubiquitously encoded housekeeping p65 gene does not contain NF-κB binding motifs in its promoter region (45), the p65 transcript has three AU-rich elements in the 3’ untranslated region, which would make the gene susceptible to regulation by RNA-stabilizing HuR protein. Therefore, when HuR protein is confined in the nuclear region through binding to ATF3, the balance between synthesis and degradation of p65 transcript in the cytoplasm would be disrupted. Prolonged presence of ATF3 in the nuclei would thus lead to depletion of cytosolic p65 transcript, which would contribute to the decreased induction of proinflammatory chemokine expression observed in the current study. As a result, ribosomal inactivation can trigger epithelial tolerance to Nod2-linked proinflammatory activation by inducing ATF3 expression. These findings suggest that ribosomal inactivation may be a potent modulator of anti-inflammatory responses to NF-κB–associated epithelial inflammatory insults.

In addition to NF-κB, Nod2-activated EGR-1 was found to be critical for chemokine gene expression in the current study. The expression of EGR-1 can be induced by a range of stimuli, which in many cases also serve as NF-κB activators (46–48). NF-κB and EGR-1 have been shown to cooperatively stimulate chemokine gene transcription (49). Moreover, the EGR-1 promoter includes an NF-κB binding motif that is important for the regulation of EGR-1 expression (50). Results from the current study suggested that ATF3 epigenetically regulated EGR-1 expression by recruiting HDAC1 and epigenetically regulate EGR-1 expression. In summary, disrupted EGR-1 expression and p65 mRNA destabilization contribute to attenuated proinflammatory chemokine production in human enterocytes.
including lymphocyte proliferation responses, monocyte maturation, and host defense against pathogens (60–63). Another recent study (62) demonstrated that ribosomal inactivation decreases the production of antibacterial mediators such as reactive oxygen species by attenuating the expression of TLRs [TLR4, TLR2, and TLR6 (62)]. In addition to altered PRR expression, reduced production of antibacterial mediators can also be caused by increased ATF3 expression, which would lead to attenuated host defense against pathogens. Moreover, the wound-healing process during mucosal inflammation can be inhibited owing to increased ATF3 expression by ribosomal stress because epithelial NF-kB and EGR-1 expression promotes the reconstitution of injured mucosal monolayers (64–66). The gastrointestinal tract is subjected to a wide variety of mucosal insults linked to several intestinal inflammatory diseases. No matter what the cause of the injuries, the mucosa usually responds rapidly by activating defense mechanisms that stimulate the repair and restoration of the mucosal lining (67). If the manufacture of early gene products, including epithelial NF-kB and EGR-1, is suppressed by ATF3 in the injured mucosal tissues (68–71), the compensatory response during wound healing would be inhibited. More studies are thus needed to determine whether ATF3-mediated gene regulation is beneficial or not under different pathogenic conditions.

As previously mentioned, Nod2 usually triggers detrimental proinflammatory responses. However, Nod2-mediated production of defense molecules can facilitate host protection against commensal bacteria and other pathogens. Absence of Nod2-linked functions observed in Crohn’s disease patients with mutations in the Nod2 gene allows increased microbial translocation and invasion into the gut submucosa, which can activate alternate PRR-linked proinflammatory pathways (6–8). Therefore, simply blocking PRR-linked signaling may not be sufficient for inhibiting proinflammatory pathways. In addition to ATF3, PPARγ expression is also induced by ribosome-inactivating DON in the presence of LPS (37, 72). PPARγ is a negative regulator of chemokine mRNA stability because this factor also suppresses the cytoplasmic translocation of HuR (73). In contrast, PPARγ-regulating C/EBP homologous protein is a positive modulator of HuR protein export from the nucleus (73). Therefore, ribosomal inactivation enhances anti-inflammatory ATF3 and PPARγ expression while paradoxically activating proinflammatory gene expression via EGR-1 and C/EBP homologous protein (33, 72). Therefore, variations in the function of signaling networks should be compared under different pathogenic conditions.

In the current study, we proposed that ATF3 is a critical regulator in the convergent signaling pathways of EGR-1 and NF-kB, and contributes to the suppression of proinflammatory gene expression promoted by Nod2. Although the mechanisms of two pathways for ATF3 downstream regulation in Nod2-activated proinflammatory signal could be different, ATF3 has been found to be an integral signal molecule that regulates proinflammatory responses triggered by Nod2 activation in the current study as well as TLR activation in human colon Caco2/bbe cells. Gastroenterology 127: 1401–1409.

Disclosures

The authors have no financial conflicts of interests.

References
