Epithelial Cholesterol Deficiency Attenuates Human Antigen R-linked Pro-inflammatory Stimulation via an SREBP2-linked Circuit*  

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Seong-Hwan Park‡, Juil Kim‡, Mira Yu‡, Jae-Hong Park‡, Yong Sik Kim‡, and Yuseok Moon‡

From the ‡Laboratory of Mucosal Exposome and Biomodulation, Department of Biomedical Sciences and Medical Research Institute, Pusan National University School of Medicine, Yangsan 50612, the ‡Department of Pediatrics, Pusan National University, Yangsan 50612, the ‡Department of Pharmacology, College of Medicine, Seoul National University, Seoul 03080, and the ‡Immunoregulatory Therapeutics Group in Brain Busan 21 Project, Busan 46241, Korea

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Patients with chronic intestinal ulcerative diseases, such as inflammatory bowel disease, tend to exhibit abnormal lipid profiles, which may affect the gut epithelial integrity. We hypothesized that epithelial cholesterol depletion may trigger inflammation-checking machinery via cholesterol sentinel signaling molecules whose disruption in patients may aggravate inflammation and disease progression. In the present study, sterol regulatory element-binding protein 2 (SREBP2) as the cholesterol sentinel was assessed for its involvement in the epithelial inflammatory responses in cholesterol-depleted enterocytes. Patients and experimental animals with intestinal ulcerative injuries showed suppression in epithelial SREBP2. Moreover, SREBP2-deficient enterocytes showed enhanced pro-inflammatory signals in response to inflammatory insults, indicating regulatory roles of SREBP2 in gut epithelial inflammation. However, epithelial cholesterol depletion transiently induced pro-inflammatory chemokine expression regardless of the well known pro-inflammatory nuclear factor-κB signals. In contrast, cholesterol depletion also exerts regulatory actions to maintain epithelial homeostasis against excess inflammation via SREBP2-associated signals in a negative feedback loop. Mechanistically, SREBP2 and its induced target EGR-1 were positively involved in induction of peroxisome proliferator-activated receptor γ (PPARγ), a representative anti-inflammatory transcription factor. As a crucial target of the SREBP2-EGR-1-PPARγ-associated signaling pathways, the mRNA stabilizer, human antigen R (HuR) was retained in nuclei, leading to reduced stability of pro-inflammatory chemokine transcripts. This mechanistic investigation provides clinical insights into protective roles of the epithelial cholesterol deficiency against excessive inflammatory responses via the SREBP2-HuR circuit, although the deficiency triggers transient pro-inflammatory signals.

Clinically, inflammatory bowel disease (IBD) patients tend to exhibit dyslipidemia, such as high levels of LDL cholesterol compared with those in healthy subjects, which is more profound in Crohn’s disease (CD) than ulcerative colitis (UC) patients (1, 2). Moreover, cholesterol depletion and subsequent lipid raft disruption in the intestinal epithelial cells are clinical features of sterol-linked inflammatory microenvironments and precede the loss of barrier function in experimental colitis (3). Mechanistically, many of the crucial tight junction molecules are located in the cholesterol-enriched lipid rafts, whose physical stability is highly affected by cellular cholesterol levels (4). Therefore, interference with cellular cholesterol levels can disorganize lipid rafts and tight junction, leading to disruption of the gut barrier. Lipid rafts are crucial to maintaining cellular integrity, epithelial polarity, and the brush border surface in intestinal epithelial cells (IECs) (5), thus suggesting some involvement in epithelial barrier-damaged diseases. IECs in experimental UC models and in intestinal biopsies of UC patients show disintegration of lipid rafts, which triggers a reduction of the tight junction-associated protein occludin and subsequently increases intestinal permeability (3). Moreover, the cholesterol-enriched lipid rafts are a platform for multiple receptor-linked signaling pathways (6, 7). These signal-associated receptors move to the lipid rafts for facilitating signal transduction and activation of effector molecules, but cholesterol depletion attenuates these cellular signals (7).

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† To whom correspondence should be addressed: Dept. of Biomedical Sciences, Pusan National University School of Medicine, Yangsan 50612, Korea. Tel.: 82-51-510-8094; Fax: 82-55-382-8090; E-mail: moon@pnu.edu.

2 The abbreviations used are: IBD, inflammatory bowel disease; 3′UTRs, 3′ untranslated regions; ABCA1, ATP-binding cassette transporter; ActD, actinomycin D; AMPK, AMP-activated kinase; ANS, anisomycin; AREs, AU-rich elements; CDK, cyclin-dependent kinases; CE, cholesterol ester; CXCL-1, C-X-C motif chemokine ligand 1; DON, deoxynivalenol; DSS, dextran sodium sulfate; E-box, enhancer box; EGR-1, early growth response-1; ER, endoplasmic reticulum; FC, free cholesterol; FXR, farnesoid X receptor; GFP, green fluorescent protein; HDL-C, high density lipoprotein cholesterol; HuR, human antigen R; IECs, intestinal epithelial cells; IHC, immunohistochemistry; LDL-C, low-density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; LXRα, liver X receptor-α; MβCD, methyl-β-cyclodextrin; NALP3, NACHT, LRR and PYD domains-containing protein 3; NF-κB, nuclear factor κB; PCSK9, proprotein convertase subtilisin/kexin type 9; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, peroxisome proliferator response element; PXR, pregnane X receptor; SCAP, SREBP cleavage-activating protein; SRE, the sterol regulatory element; SREBP, the sterol regulatory element-binding protein; TC, total cholesterol; TJ, tight junction; UC, ulcerative colitis.
The human intestine plays a key role in cholesterol balance because it is the only site for absorption of dietary sterols and quantitatively represents the single most active location of cholesterologenesis (8, 9). Cholesterol depletion triggers synthesis of cholesterol to maintain cholesterol homeostasis in IECs. Among the various intracellular modulators of lipid homeostasis, sterol regulatory element (SRE)-binding proteins (SREBPs) are the major transcription factors for cholesterol synthesis, and many studies support the relationship between SREBPs and cholesterol synthesis (10). SREBP1α and SREBP1c are derived from the same gene; however, SREBP1α is an activator associated with biosynthesis of both cholesterol and fatty acids, whereas SREBP1c tends to be more active in up-regulation of fatty acid synthesis (11). SREBP2 is an important molecule for inducing cholesterol synthesis and regulates target genes, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), a rate-limiting enzyme in the cholesterol biosynthesis pathway (12). In addition to cholesterol biosynthesis, SREBP2 is also associated with expression of NPC1L1 as a positive transcription factor by directly binding to the NPC1L1 promoter for absorption of cholesterol (13, 14). Endoplasmic reticulum (ER)-anchored SREBP2 is activated by SREBP cleavage-activating protein (SCAP), which senses internal cholesterol levels and escorts SREBP2 to the Golgi body via formation of the SCAP-SREBP complex (15). In the Golgi body, site-1 protease and site-2 protease, a membrane-bound serine protease, cleave SREBP2, and the N terminus of SREBP2 translocates to the nucleus and induces the expression of target genes as a transcription factor (16). Therefore, SREBP2 is a crucial cholesterol sensor that is proteolytically released from ER membranes and acts as a regulator of intracellular cholesterol synthesis. The mucosal epithelia, as the first line of defense against various luminal insults, including nutrients and pathogens, transfer the initial immunological recognition signals to the submucosal immune systems (17). Because cholesterol is crucial for epithelial barrier integrity and cellular signaling regulation via lipid rafts as key signal-modulating domains, we assessed the effects of cholesterol depletion on the innate immune signals in human enterocytes. We hypothesized that epithelial cholesterol deficiency may trigger inflammation-checking machinery via cholesterol sentinel signaling molecules. In the present study, SREBP2 as the cholesterol sentinel was assessed for its involvement in the epithelial inflammatory responses, which will provide new clinical insights into epithelial cholesterol-linked intervention in the mucosa-associated disorders, such as IBD and inflammation-associated intestinal malignancy.

**Results**

**SREBP2 Expression Is Attenuated in Inflamed Enterocytes**—As a sterol sentinel during the inflammatory insults, SREBP2 was measured in the inflamed colon of IBD patients and mice with chemical-induced colitis. SREBP2 expression was suppressed in the inflamed colonic tissue of DSS-treated mice compared with control group colonic tissue (Fig. 1A). Because male mice are more susceptible to lethality by DSS-induced gastrointestinal ulceration, the female murine ulcer models are generally used to investigate the active biological events during inflammation. However, SREBP2 suppression was also observed in the male mice exposed to DSS (data not shown). Besides, SREBP2 activation was also suppressed in DSS-treated female mice (Fig. 1B). However, tissue and serum cholesterol levels were increased in DSS-treated mice (Fig. 1, C and D). In patients with Crohn’s disease, SREBP2 expression was also significantly reduced in inflamed tissue compared with normal parts (Fig. 1E). To confirm the roles of SREBP2 in the pro-inflammatory signaling pathway, SREBP2-deficient enterocytes were assessed for chemokine induction. In IBD patients, monocyte-derived IL-1β and TNF-α are abundant in the intestinal tissue and mediate intestinal inflammation (18, 19). We investigated the roles of SREBP2 in pro-inflammatory signals in vitro using HCT-8 human intestinal epithelial cells in the presence of IL-1β or TNF-α because SREBP2 knock-out mice are lethal. The HCT-8 cell line is one of the widely used enterocyte models to study inflammatory diseases and microbial infection (20, 21). Besides, the ileocecum of the small intestine, which is the source of HCT-8 cells, is one of the most susceptible to cholesterol metabolism. TNF-α or IL-1β induced the expression of pro-inflammatory chemokines, such as IL-8 (Fig. 1, F and G) and CXCL-1 (Fig. 1, H and I), which were more enhanced in SREBP2-suppressed enterocytes compared with the control cells. Taken together, epithelial SREBP2 plays regulatory roles in pro-inflammatory activation, and the status of suppressed SREBP2 and increased cholesterol contents in inflamed colonic tissue can account for the exacerbated epithelial inflammation in animals and patients with colitis as demonstrated in Fig. 1, A and B.

**SREBP2 Down-regulates Chemokine Induction in Cholesterol-depleted Enterocytes—Disruption of cellular cholesterol homeostasis using methyl-β-cyclodextrin (MβCD) was assessed for effects on pro-inflammatory chemokine expression in enterocytes. Cholesterol depletion was positively associated with chemokine induction in enterocytes (Fig. 2A). In contrast, cholesterol supplement increased the intracellular levels of cholesterol (total cholesterol (TC), free cholesterol (FC), and cholesterol ester (CE)) (Fig. 2B) and attenuated MβCD-triggered chemokine mRNA expression and secretion (Fig. 2, C and D), indicating that enhanced pro-inflammatory responses are due to cholesterol depletion. Similar patterns were also demonstrated in other human intestinal cell lines, including intestine 407 and HCT-116 (Fig. 2, E and F, respectively). Consistent with these data, cholesterol depletion induced mRNA expression of chemokines such as CXCL-1 and CXCL-2 in primary mouse enterocytes, which was also attenuated by cholesterol supplementation (Fig. 2G). In response to MβCD exposure in enterocytes, SREBP2 was highly activated due to loss of cholesterol; thus, supplementing with cholesterol attenuated SREBP2 activation in the cholesterol-depleted intestinal epithelial cells (Fig. 2H). Moreover, the effects of SREBP2 on pro-inflammatory signals and intracellular cholesterol levels were assessed in the cholesterol-depleted enterocytes. SREBP2 deficiency enhanced intracellular cholesterol contents (total cholesterol, free cholesterol, and cholesteryl ester) (Fig. 2I). In addition, MβCD-triggered chemokine mRNA expression was also up-regulated by SREBP2 suppression (Fig. 2J), whereas SREBP2 overexpression down-regulated chemokine expression (Fig. 2K) and IL-8 secretion (Fig. 2L), supporting the assumption that...
SREBP2 plays a regulatory role in the pro-inflammatory response to cholesterol depletion. 

* SREBP2 Promotes Expression of the Anti-inflammatory Transcription Factor Peroxisome Proliferator-activated Receptor γ (PPARγ) via an Early Growth Response-1 (EGR-1)-linked Pathway in Cholesterol-depleted Enterocytes—As a representative anti-inflammatory transcription factor, PPARγ regulates pro-inflammatory signals both at transcriptional and post-transcriptional levels (22). PPARγ was thus assessed for its involvement in cholesterol depletion-induced chemokine production. Based on the assumption that SREBP2 regulates pro-inflammatory signals via PPARγ in the present study, we observed the effects of SREBP2 deficiency on PPARγ expression and activation in cholesterol-depleted enterocytes. Cholesterol depletion increased PPARγ expression and transcriptional activity of the PPARγ-binding promoter, all of which were suppressed by knockdown of SREBP2 using shRNA (Fig. 3, A and B). Moreover, induction of PPARγ by cholesterol depletion was attenuated by cholesterol supplementation in enterocytes (Fig. 3C), indicating the cholesterol-mediated regulation of PPARγ. Because SREBP2 counteracted the cholesterol depletion-induced chemokine production, SREBP2-promoted PPARγ was also assessed for its regulatory roles in pro-inflammatory chemokine production. As expected, suppression of PPARγ action by expressing its dominant negative form eventually enhanced cholesterol depletion-induced chemokine expression (Fig. 3, D and E), whereas enhancement of PPARγ action by expressing its wild type form suppressed the chemokine expression (Fig. 3, F and G). In addition to PPARγ, other anti-inflammatory nuclear receptors, including pregnane X recep-
FIGURE 2. Cholesterol depletion-induced chemokine and SREBP2 expression in enterocytes. A, HCT-8 cells were treated with vehicle or 1 mM MβCD for the indicated times, and mRNA was quantified. * or #, significant differences from each 0 h control group (p < 0.05). B, HCT-8 cells were treated with vehicle or 20 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 4 h. Intracellular total cholesterol (TC), free cholesterol (FC), or cholesteryl ester (CE) contents were measured by a fluorometric assay. Different letters (a–c) over each bar represent significant differences between groups (p < 0.05). C, HCT-8 cells were treated with vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 4 h, and mRNA was quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). D, HCT-8 cells were treated with vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 4 h, and mRNA was quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). E and F, intestine 407 (I) and HCT-116 (P) cells were treated with the vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 4 h, and mRNA was quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). G, the isolated mouse enterocytes were treated with vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 4 h, and mRNA was quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). H, HCT-8 cells were treated with the vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 1 h for protein analysis. I, HCT-8 cells were transfected with control or shSREBP2. Intracellular total cholesterol (TC), free cholesterol (FC), or cholesteryl ester (CE) contents were measured by a fluorometric assay. Asterisks indicate significant differences between control and shSREBP2-transfected cells (*, p < 0.05; **, p < 0.01). J, HCT-8 cells transfected with the control vector or shSREBP2 were exposed to the vehicle or 1 mM MβCD for 4 h. IL-8 and CXCL-1 mRNA were quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). K, HCT-8 cells transfected with the control vector or SREBP2-FLAG were exposed to the vehicle or 1 mM MβCD for 4 h. IL-8 and CXCL-1 mRNA were quantified. Different letters (a–c) over each bar represent significant differences between groups (p < 0.05). L, IL-8 secretion was assessed. First, EGR-1 expression was induced by cholesterol depletion using ELISA. Different letters (a–c) over each bar represent significant differences between groups (p < 0.05). A–J, all results are representative of three independent experiments. Error bars, S.D.

Cholesterol-linked Regulation of Pro-inflammatory Signals

Because the sterol response element for SREBP2 binding does not exist in the PPARγ promoter, it may be speculated that another transcription factor mediates SREBP2-induced PPARγ expression. Among the genes with the SRE-inclusive promoter, early growth response-1 (EGR-1) is well known as a positive regulator of PPARγ expression by binding directly to the PPARγ promoter at EGR-1 binding sites overlapped with Sp1 (23). Assuming that EGR-1 mediates SREBP2-induced PPARγ expression, the roles of EGR-1 in cholesterol-depleted enterocytes were assessed. First, EGR-1 expression was induced by cholesterol depletion, which was attenuated by cholesterol sup-
plementation both at the protein and mRNA levels (Fig. 4, A and B, respectively). In addition, we assessed the effects of EGR-1 suppression on PPARγ mRNA expression and PPARγ promoter-binding activity in enterocytes. Genetic knockdown of EGR-1 using its shRNA decreased lipid cholesterol depletion-induced PPARγ mRNA expression (Fig. 4C). Enhanced binding of EGR-1 to PPARγ promoter in response to cholesterol depletion was decreased in EGR-1-deficient cells (Fig. 4D). Moreover, PPARγ binding activity to the consensus promoter of its target genes was also elevated by cholesterol depletion, which was alleviated by EGR-1 suppression (Fig. 4E). Contrary to the PPARγ promoter, the EGR-1 promoter contains several SREBP family protein-binding sites, including SRE and a classic palindromic enhancer box (E-box) (24). Therefore, we examined how EGR-1 expression was regulated by cholesterol depletion-activated SREBP2. Cholesterol depletion-induced EGR-1 expression was reduced by SREBP2 deficiency (Fig. 4F). In addition to protein expression, cholesterol depletion increased the transcriptional activity of EGR-1, which was mitigated by SREBP2 deficiency (Fig. 4G). In particular, SREBP2 was found to bind to the EGR-1 promoter (Fig. 4H). Functionally, PPARγ-enhancing EGR-1 was thus a negative regulator of the pro-inflammatory chemokines, such as IL-8 and CXCL-1, in cholesterol-depleted enterocytes (Fig. 4, I and J). Taken together, cholesterol depletion-activated SREBP2 was positively involved in transcriptional activation of EGR-1, and subsequently
enhanced EGR-1 was the positive regulator of PPARγ induction in response to cholesterol depletion, all of which act as the regulatory machinery against pro-inflammatory signals.

Cholesterol Depletion-induced Pro-inflammatory Chemokine Expression Is Mediated by the RNA-binding Protein HuR and Not by Nuclear Factor κB (NF-κB) Transcription Activity in Human Enterocytes—Cholesterol depletion activates various pro-inflammatory response-associated signals, such as NF-κB, a central transcription factor in inflammation (25). Therefore, we assessed the involvement of NF-κB activation to promote expression of genes for pro-inflammatory chemokines in response to cholesterol depletion. FLAG-tagged SR-IκB construct, which expresses a mutated form of IκBα that cannot be phosphorylated and degraded, was used to inhibit NF-κB translocation into the nucleus. Cholesterol depletion-induced chemokine mRNA and its promoter activity were not significantly blocked by NF-κB inhibition, and phosphorylation of p65, a NF-κB subunit, was not altered by cholesterol depletion in the present model (data not shown), suggesting the presence of additional mechanisms for chemokine expression other than...
transcriptional activation. Next, we checked the effect of cholesterol depletion on the stability of chemokine mRNA in enterocytes. The half-lives of IL-8 and CXCL-1 mRNA were significantly enhanced by cholesterol depletion, which suggests the involvement of post-transcriptional regulation in chemokine induction (Fig. 5, A and B). As mediators of post-transcriptional regulation, RNA-binding proteins may play important roles in cholesterol depletion-associated mRNA stabilization. Although most RNA-binding proteins destabilize targeted mRNA, a few of these proteins, including human antigen R (HuR), bind to 3′-UTRs with AU-rich elements and stabilize the transcripts, including ones encoding pro-inflammatory cytokines (26). In the present study, the impact of HuR on chemokine mRNA stabilization was assessed in cholesterol-depleted enterocytes. Blocking the HuR protein using its shRNA almost completely suppressed the IL-8 and CXCL-1 induced by cholesterol depletion, indicating the positive regulation of HuR in chemokine induction (Fig. 5, C and D). Moreover, cholest-

FIGURE 5. Roles of HuR in cholesterol depletion-mediated stabilization of pro-inflammatory chemokines. A and B, HCT-8 cells were exposed to vehicle or 1 mM MβCD for 4 h. Cellular transcription was then arrested by adding 5 μM actinomycin D. IL-8 (A) and CXCL-1 (B) mRNA were quantified. *, significant differences from the vehicle group at each indicated time (p < 0.05). C and D, the control vector- or shHuR-expressing HCT-8 cells were treated with vehicle or 1 mM MβCD for 4 h. IL-8 (C) and CXCL-1 (D) mRNA were quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). E, HCT-8 cells were treated with 1 mM MβCD for the indicated times. Cytosolic and nuclear fractions of cell lysates were subjected to Western blotting analysis. F, HCT-8 cells were treated with vehicle or 1 mM MβCD for 4 h. HuR protein was visualized with anti-HuR antibody (top). Bottom, relative quantitative values of cytosolic HuR. The ratio indicates the measured nuclear densities of HuR signal in the cytoplasm outside of the DAPI-stained area. ***, significant differences compared with the vehicle group (p < 0.001). G, HCT-8 cells were treated with vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 4 h. Cytosolic and nuclear proteins were analyzed. H and I, control vector- or shHuR-expressing HCT-8 cells were treated with 1 mM MβCD for 4 h. Cellular transcription was then arrested by adding 5 μM actinomycin D. IL-8 (H) and CXCL-1 (I) mRNA were quantified. *, significant difference from the vehicle group at each indicated time (p < 0.05). J, a plasmid containing a CMV promoter-linked reporter gene tagged with the 3′-UTR of the human IL-8 gene was constructed (top). The control or shHuR-expressing HCT-8 cells transfected with the reporter plasmid were treated with vehicle or 1 mM MβCD for 12 h, followed by measurement of luciferase activity (bottom). Different letters (a–c) over each bar represent significant differences between groups (p < 0.05). K and L, HCT-8 cells transfected with the control vector or shHuR were treated with vehicle or 1 mM MβCD for 4 h. RNA-bound HuR was immunoprecipitated, and the levels of HuR-bound IL-8 (K) and CXCL-1 (L) mRNA were quantified. Different letters (a–f) over each bar represent significant differences between groups (p < 0.05). n.s., not significant (A–L). All results are representative of three independent experiments. Error bars, S.D.
terol depletion triggered the cytosolic translocation of HuR protein from the nucleus to the cytoplasm, which was maintained for more than 12 h (Fig. 5E). Cytosolic translocation of the HuR protein was also observed and quantified under the confocal microscope (Fig. 5F). However, cholesterol supplementation alleviated the cytosolic translocation of HuR protein in response to cholesterol depletion (Fig. 5G). Because we confirmed that cholesterol depletion mediated HuR protein translocation to cytoplasm, we assessed the effects of HuR protein on mRNA stabilization of cholesterol depletion-induced pro-inflammatory chemokines. The half-lives of IL-8 and CXCL-1 mRNA induced by cholesterol depletion were shortened by HuR suppression using its shRNA in human enterocytes (Fig. 5, H and I). Because the 3′-UTR of IL-8 mRNA contains 10 AU-rich elements (AREs), we constructed a CMV promoter-activated reporter gene linked with the 3′-UTR of the IL-8 gene to observe the contribution of HuR protein to ARE-related mRNA stability (27). Cholesterol depletion enhanced the reporter luciferase expression, which was attenuated by blocking of HuR expression using shRNA (Fig. 5J), indicating the importance of ARE-containing 3′-UTR in up-regulation of IL-8 mRNA stabilization. Moreover, cytoplasmic interaction between HuR protein and chemokine transcripts was enhanced by cholesterol depletion, which was alleviated in HuR-suppressed cells (Fig. 5, K and L). In sum, the HuR protein was translocated to the cytoplasm by cholesterol depletion, contributing to enhanced stabilization of chemokine mRNA via interaction with ARE-containing chemokine transcripts in enterocytes.

**SREBP2 Down-regulates HuR-mediated mRNA Stabilization in Cholesterol-depleted Enterocytes**—As demonstrated, SREBP2, PPARγ, and EGR-1 were involved in the negative regulation of pro-inflammatory signaling in cholesterol-depleted enterocytes, which was confirmed in terms of IL-8 production in specific gene-attenuated cells (Fig. 6A). In contrast, the RNA stability-regulating factor HuR was positively involved in IL-8 secretion in IECs with cholesterol depletion. In particular, it is reported that cytoplasmic translocation of the nuclear HuR protein is retarded by specific binding to the nuclear PPARγ (28). Moreover, cholesterol depletion-triggered translocation of HuR protein from the nuclei to the cytoplasm was also negatively regulated by PPARγ and its upstream modulators, including SREBP2 and EGR-1 (Fig. 6B). In particular, exogenously introduced PPARγ interfered with cholesterol deficiency-induced HuR translocation to cytoplasm via enhanced nuclear interaction between PPARγ and HuR (Fig. 6C). However, the translocation of HuR was marginally elevated by suppression of PXR or FXR, although these anti-inflammatory nuclear receptors were negatively involved in chemokine induction by cholesterol deficiency (Fig. 6, D and E). These suppressive roles of PPARγ and its upstream modulators in HuR translocation were further assessed using confocal analysis (Fig. 6F). Because cytosolic HuR protein can bind to AREs in the 3′-UTR of IL-8 and CXCL-1 mRNA, we assessed the amount of IL-8 and CXCL-1 mRNA bound to HuR protein in the cytoplasm. Genetic knockdown of PPARγ, SREBP2, or EGR-1 increased the cytosolic IL-8 and CXCL-1 mRNA bound to HuR protein in cholesterol-depleted enterocytes (Fig. 6, G and H), suggesting that SREBP2-mediated signals, including EGR-1 and PPARγ, are negative regulators of HuR translocation to the cytoplasm in IECs. Next, SREBP2-activated signals were assessed for their effects on HuR-mediated stabilization of ARE-containing transcripts. Suppression of SREBP2-activated signals enhanced the stability of cholesterol depletion-induced IL-8 and CXCL-1 mRNA (data not shown). These results suggest that cholesterol depletion-activated SREBP2 signals, including EGR-1 and PPARγ, are negative regulators of cytoplasmic translocation of HuR protein, leading to suppression of cholesterol depletion-induced chemokine expression.

**Cholesterol Depletion Attenuated HuR-mediated Pro-inflammatory Stimulation in SREBP2-linked Pathways**—As suggested, cholesterol depletion enhanced the pro-inflammatory signaling responses via HuR protein, but the subsequent excessive inflammatory responses were repressed in a feedback pathway via SREBP2-linked signals. We confirmed the effects of pre-exposure to cholesterol depletion on HuR-mediated inflammatory responses in human IECs. As well established HuR triggers, ribosome-inactivating agents, such as deoxynivalenol (DON) and anisomycin (ANS), which bind to the 28S ribosomal RNA peptidyltransferase site and block protein synthesis, were used to specifically induce HuR-mediated chemokine production (27). Pretreatment with cholesterol-depleting MβCD suppressed the ribosome-inactivation-induced expression of IL-8 and CXCL-1 mRNA, which was attenuated by supplementing with cholesterol (Fig. 7, A and B). We then tested whether ribosome inactivation-induced chemokines were dominantly mediated by HuR protein (Fig. 7, C and D). Moreover, ribosome-inactivation triggered the cytosolic translocation of nuclear HuR protein, which was retarded by cholesterol depletion (Fig. 7E). However, cholesterol supplementation restored the HuR protein translocation to the cytoplasm (Fig. 7E). Mechanistically, HuR-mediated chemokine production after pre-exposure to cholesterol depletion was assessed in the previously suggested regulatory signaling pathway, the SREBP2-ERG-1-PPARγ circuit. Genetic knockdown of SREBP2, EGR-1, or PPARγ restored the cholesterol depletion-suppressed chemokine expression (Fig. 7, F and G), indicating the negative regulation of chemokine expression by these three transcription factors. In conclusion, cholesterol deficiency-activated SREBP2-linked signals induced epithelial tolerance to HuR-mediated gene regulation. In agreement with results in the cell-based assessment, the HuR regulation in SREBP2-ERG-1-PPARγ signal was confirmed in the insulited colon of mice with colitis. It has been well known that PPARγ expression is down-regulated in DSS-induced colitis model and human tissues with IBD (29, 30). In addition to PPARγ suppression, the DSS treatment model in the present study also showed reduction of EGR-1 (Fig. 8A) and SREBP2 (Fig. 1A) in the insulited mouse colon. However, in response to the reduced SREBP2-ERG-1-PPARγ signal, HuR protein levels were more enhanced by DSS treatment (Fig. 8B), suggesting the potential negative regulation of HuR-linked inflammatory response by an SREBP2-ERG-1-PPARγ-associated circuit.

**Discussion**

In the present study, cholesterol depletion regulated the pro-inflammatory signals via activated SREBP2 in a feedback path-
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Way. In particular, cholesterol depletion-activated SREBP2 promoted EGR-1 expression, and the enhanced EGR-1 was positively involved in induction of PPARγ/H9253, a representative anti-inflammatory transcription factor. As a target of the SREBP2-EGR-1-PPARγ-associated signaling pathways, the transcript stabilizer HuR protein was spatially restricted in nuclei under the stress of cholesterol depletion. HuR is generally characterized as a stabilizer of AU-rich element-containing transcripts, including IL-8 and CXCL-1 mRNA. Cholesterol depletion-induced SREBP2-linked signals retarded the cytosolic translocation of HuR protein, leading to reduction in stability of chemokine mRNA. Although the epithelial cholesterol deficiency transiently induced chemokine expression, it triggered SREBP2-linked inflammation-checking machinery via regulation of HuR, eventually leading to tolerance to pro-inflammatory events (Fig. 8C). In terms of nutrition, dietary restriction of cholesterol could be thus beneficial by triggering the SREBP2-linked anti-inflammatory actions in the gut barrier. However, because the cholesterol deficiency can also promote transient induction of chemokines, cholesterol-bypassing acti-

FIGURE 6. Effect of cholesterol-depleted activated SREBP2-linked signals on HuR translocation and subsequent binding to chemokine transcripts. A, the control vector, shHuR, shSREBP2, shEGR-1- or PPARγ DN-transfected HCT-8 cells were treated with vehicle or 1 mM MβCD for 24 h. IL-8 secreted into the culture medium was quantified using ELISA. Different letters (a–h) over each bar represent significant differences between groups (p < 0.05). B, the control vector, shSREBP2, shEGR-1-, or PPARγ DN-transfected HCT-8 cells were treated with vehicle or 1 mM MβCD for 4 h. Cytosolic and nuclear fractions of cell lysates were subjected to Western blotting analysis. C, the control or PPARγ WT-transfected HCT-8 cells were treated with vehicle or 1 mM MβCD for 4 h. Cytosolic and nuclear fractions of cell lysates were subjected to Western blotting analysis. Cellular nuclear lysates were also immunoprecipitated with anti-FLAG antibody. The precipitated samples were subjected to Western blotting analysis. D and E, HCT-8 cells transfected with control vector, shFXR (E), or shFXR (E) were treated with vehicle or 1 mM MβCD for 4 h. Cytosolic and nuclear fractions of cell lysates were subjected to Western blotting analysis. The relative quantitative values of cytosolic HuR. The ratio is the measured densities of HuR signals outside of the DAPI-stained area. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). The box graph shows the relative quantitative values of IL-8 (G) or CXCL-1 (H) mRNA were quantified. Different letters (a–g) over each bar represent significant differences between groups (p < 0.05). The box graph shows levels of cytosolic HuR protein that were analyzed using Western blotting analysis. All results are representative of three independent experiments. Error bars, S.D.
vation or induction of SREBP2 would be a promising intervention against the inflammatory insults in the patents with chronic intestinal inflammation.

In this study, SREBP2 was the key molecule to regulate the expressions of EGR-1 and PPARγ, all of which subsequently counteracted the cholesterol depletion triggering of pro-inflammatory signals by interfering with the action of cytoplasmic HuR protein. Because cholesterol depletion itself can also trigger inflammatory responses, activation of SREBP2 by cellular cholesterol depletion is not a sound method to attenuate HuR-linked responses in the gut. Instead, specific modulation of SREBP2 activity can warrant the target of intervention with the HuR-mediated intestinal epithelial inflammation and related regulation of HuR-linked pathogenesis without stimulation of pro-inflammatory responses. However, SREBP2-mediated anti-inflammatory responses are not always beneficial to the host because these can weaken the defense against infectious agents. For instance, whereas SREBP2 activation can offer protection against inflammatory insults, SREBP2-linked cholesterol synthesis is crucial for some viral infections (31), indicating that SREBP2 increases susceptibility to secondary infections during infection-associated immune suppression. Therefore, because the regulation of SREBP2-linked anti-inflammatory action has both good and bad effects, careful assessment is required for better therapeutic application against metabolism-associated inflammatory diseases.

FIGURE 7. Cholesterol depletion regulates HuR-mediated chemokine induction. A and B, HCT-8 cells were pre-exposed to vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 18 h. The cells were then treated with vehicle, 500 ng/ml DON, or 50 ng/ml ANS for 1 h. IL-8 (A) and CXCL-1 mRNA (B) mRNA were quantified. Different letters (a–f) over each bar represent significant differences between groups (p < 0.05). C and D, HCT-8 cells transfected with the control vector or shHuR were treated with vehicle, 500 ng/ml DON, or 50 ng/ml ANS for 1 h. IL-8 and CXCL-1 mRNA were quantified. Different letters (a–d) over each bar represent significant differences between groups (p < 0.05). E, HCT-8 cells were pretreated with vehicle or 1 mM MβCD in the absence or presence of 20 μg/ml cholesterol for 18 h. The cells were then treated with vehicle, 500 ng/ml DON, or 50 ng/ml ANS for 1 h. Cytosolic and nuclear fractions of cell lysate were subjected to Western blotting analysis. F and G, HCT-8 cells transfected with the control vector, shSREBP2, shEGR-1, or PPARγ DN were pre-exposed to vehicle or 1 mM MβCD for 18 h. The cells were then treated with vehicle, 500 ng/ml DON, or 50 ng/ml ANS for 1 h. IL-8 (F) and CXCL-1 (G) mRNA were quantified. Different letters (a–i) over each bar represent significant differences between groups (p < 0.05) (A–G). All results are representative of three independent experiments. Error bars, S.D.
In terms of signal transduction, cholesterol depletion-activated SREBP2 showed anti-inflammatory action against excessive production of pro-inflammatory chemokines via EGR-1 and PPARγ. However, in some studies, SREBP2 is a positive regulator of *IL-8* expression by binding to a consensus sequence SRE located in the *IL-8* promoter (−134 to −125) (32) and triggers IL-1β-mediated inflammation via formation of NALP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasome (33). In contrast to these reports, SREBP2 activation did not contribute to transcription of chemokine expression in cholesterol-depleted enterocytes in the present study. Generally, SREBPs interfere with the actions of PXR or FXR on gene transcription (34, 35). As anti-inflammatory nuclear receptors, FXR and PXR were negatively involved in chemokine induction by cholesterol deficiency, but they did not affect the cytoplasmic translocation of HuR protein, which was a critical stabilizer of chemokine transcripts in the present study. However, anti-inflammatory action of SREBP2 in the present study was rather due to its positive regulation of PPARγ expression and subsequent nuclear restriction of HuR protein, leading to reduced stabilization of chemokine transcripts. Moreover, NF-κB, one of the most well known pro-inflammatory transcription factors, was not involved in chemokine induction by cholesterol depletion. Instead, SREBP2 exerts suppression of chemokine expression by destabilization of *IL-8* and *CXCL-1* mRNA via regulation of HuR localization. Although SREBP2 could promote *IL-8* transcription as reported in the previous study (32), the stability of transcribed mRNA of *IL-8* was
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decreased by restricted availability of cytosolic HuR, which was caused by nuclear sequestering via SREBP2/EGR-1-induced PPARγ. In addition to regulation of cytoplasmic translocation of HuR by PPARγ in human enterocytes, shuttling of HuR protein across the nuclear membrane is regulated by other signaling molecules, including different members of the MAPK family, the AMPK (AMP-activated kinase) family, cyclin-dependent kinases (CDK1 or -2), and PKC. In particular, p38 MAPK and PKCα/δ enhance the ATP-dependent export of HuR protein via phosphorylation (36, 37). However, because PKC is positively involved in ribosome inactivation-triggered HuR translocation in enterocytes (27) and PPARγ inhibits the PKC signaling cascade by limiting membrane translocation (38), it is appropriate to speculate that cytosolic translocation of HuR is mediated by the PKC signaling pathway, which might be inhibited by PPARγ activation.

In addition to the functions of epithelial cholesterol in the regulation of inflammatory responses in the gut, the cholesterol deficiency may influence the metabolic processes in the intestine because the small intestine is one of the key organs for cholesterol balance and represents the single most active location of cholesterologenesis, as mentioned in the Introduction. In response to the cholesterol depletion, PPARγ is also known to be a crucial sterol sensor like SREBP2 that plays central roles in cholesterol homeostasis. Whereas SREBP2 is crucial for cholesterol synthesis, PPARγ agonists increase cholesterol efflux by attenuating the expression of ATP-binding cassette transporter (ABCA1), a cholesterol efflux pump, and liver X receptor-α (LXRXα) in the cellular lipid removal pathway via inhibition of miR-613 expression by targeting the 3′-UTRs of ABCA1 and LXRXα mRNA (39). Moreover, PPARγ regulates SREBP2-dependent cholesterol synthesis and uptake in both liver and intestinal epithelial cells (40). PPARγ activation down-regulates the action of SREBP2, leading to a reduction of SREBP2-targeted HMG-CoA reductase and LDL receptor (LDLR) mRNA expression, which are related to disrupted cholesterol homeostasis in the liver and IECs (40). In the present study, cellular cholesterol depletion was found to activate SREBP2, which was positively associated with induction of PPARγ. PPARγ is thus supposed to counteract actions of SREBP2-altered lipids, resulting in maintenance of the lipid homeostasis. In addition to PPARγ, SREBP2-induced EGR-1 can be involved in cholesterol homeostasis. EGR-1 may decrease cholesterol absorption because EGR-1 is a regulator of LDLR, a crucial cholesterol transporter in circulation (41). SREBP2 is the positive transcriptional regulator of proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 post-translationally regulates LDLR by binding to LDLR on the cell surface, leading to LDLR degradation and subsequently reduction in cellular uptake of cholesterol (42). In the present model, SREBP2 suppression decreased PCSK9 expression with enhanced levels of LDLR protein (data not shown). This network can account for the slight increase of cellular cholesterol in response to SREBP2 suppression in the present model (Fig. 2I). Therefore, the SREBP2-linked circuit as the inflammation-checking machinery contains more complicated cross-talk among components in the circuit that modulates cellular lipid profiles in the intestine, a key player of cholesterol homeostasis.

The critical factor mediating mucosal immune tolerance is PPARγ. Moreover, PPARγ expression is also reduced in IBD patients, particularly in the colonic epithelial cells (43). PPARγ has been investigated as a critical regulator of gut homeostasis because epithelial PPARγ activation generally reduces gene expression of pro-inflammatory mediators (44). Genetic ablation of PPARγ is observed in patients with ulcerative colitis, which is associated with severe chronic inflammatory outcomes (45). When expressed in the gut epithelium, PPARγ thus protects against excessive colonic inflammatory responses to both commensal and pathogenic insults (46). The most widely accepted target of action of PPARγ is pro-inflammatory NF-κB in leukocytes, and the attenuation of epithelial inflammatory response by commensal bacteria is also mediated by regulation of nuclear-cytosolic anti-directional shuttling of PPARγ and NF-κB protein (47). However, in the present study, PPARγ is a regulator of the stabilization of pro-inflammatory chemokine mRNA via inhibition of HuR shuttling between the nuclei and the cytoplasm in enterocytes under the stress of cholesterol depletion. Therefore, cellular cholesterol depletion attenuates the excessive inflammatory stimulation in enterocytes, and SREBP2-linked PPARγ can thus be regarded as a promising therapeutic target of the inflammation in metabolically insulted epithelium.

In conclusion, cholesterol depletion enhanced pro-inflammatory chemokines via transcript stabilization with HuR protein. However, this cellular cholesterol deficiency activated SREBP2-linked signaling molecules, including EGR-1 and PPARγ, all of which contributed to the limited availability of cytosolic HuR protein and subsequently attenuated the stability of the pro-inflammatory chemokine transcripts in IECs. Cholesterol deficiency in the enterocytes under the inflammatory stress may thus retard excessive inflammatory responses via an SREBP2-linked circuit. Modulation of this SREBP2-linked circuit would provide new insight into epithelial cholesterol-based intervention with gut inflammatory diseases, which should be clinically addressed in the future studies.

Experimental Procedures

Ethics Statement—This study was conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All animal experiments were also approved by the institutional animal care and use committee of Pusan National University (approval number PNU-2010-000189).

Cell Culture Conditions and Reagents—Intestinal cancer cell lines, including HCT–8 (passage 13), HCT–116 (passage 32), and intestine 407 (passage 10), were purchased from the ATCC (Manassas, VA). All cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin (Welgene, Daegu, Korea) in a 5% CO2 humidified incubator at 37°C. Additionally, the medium for the intestine 407 cells was supplemented with 1% nonessential amino acids (Invitrogen). During the xenobiotic treatment, cells were cultured in serum-free RPMI1640 medium. Cell number was assessed by trypsin blue dye exclusion (Sigma-Aldrich) using a hemocytometer. MβCD,
actinomycin D, DON (97.6 ± 2.4% pure) isolated from Fusarium graminearum, and ANS were obtained from Sigma-Aldrich. Cholesterol was purchased from Bio Basic Canada Inc. (Amherst, NY).

**Construction of Plasmids**—CMV-driven shRNA was constructed by inserting shRNA into a pSilencer 4.1-CMV-neo vector (Ambion, Austin, TX). The negative control vector, shRNA of SREBP2, EGR-1, and HuR insert-containing vector were named as control, shSREBP2, shEGR-1, and shHuR, respectively. The negative control siRNA template sequence lacks significant homology to the mouse, human, and rat genome databases, and pSilencer 4.1-CMV-neo containing the negative control siRNA template was provided by Ambion. The inserted SREBP2, EGR-1, HuR, FXR, and PXR shRNA targeted the sequences 5′-GGC TTT GAA GAC GAA GCT A-3′, 5′-GGC TTT GAA GAC GAA GCT A-3′, and 5′-GGC TTT GAA GAC GAA GCT A-3′, respectively. FLAG-tagged dominant negative PPARγ (PPARγ DN) and wild type PPARγ (FLAG-PPARγ WT) plasmids were provided by Dr. Krishna Chatterjee (University of Cambridge). The SR-1κBα-FLAG expression vector has been described previously (48).

**Luciferase Assay**—Cells were transfected with cold PBS, lysed with passive lysis buffer (Promega), and centrifuged at 13,475 × g for 10 min. The supernatant was collected, isolated, and stored at −80 °C until assessed for luciferase activity. Measurement of luciferase activity was described previously (22).

**Western Immunoblotting Analysis**—Levels of protein expression were compared by Western immunoblotting analysis using rabbit polyclonal anti-actin antibody, rabbit polyclonal anti-p65, rabbit polyclonal anti-SREBP2 rabbit polyclonal anti-EGR-1, mouse monoclonal anti-hnRNP, and mouse monoclonal anti-HuR (Santa Cruz Biotechnology, Inc.); rabbit polyclonal IκBα and rabbit polyclonal phospho-κBp65 (Cell Signaling Technology, Beverly, MA); and mouse monoclonal anti-FLAG (Sigma-Aldrich). The process of Western blotting analysis was described previously (50).

**IL-8 ELISA**—IL-8 from cell supernatants was quantified using ELISA. HCT8 cells were plated at 5 × 10⁴ cells/well of a 24-well plate. After treatment with DON or vehicle, cell culture medium was collected, and cell debris was removed by centrifugation. IL-8 levels were determined by ELISA using an OptEIA human IL-8 ELISA kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, the capture antibody was coated onto the wells of ELISA plates overnight at 4 °C. After washing with Tween 20-containing PBS and blocking with PBS supplemented with 100 g/L FBS overnight at 4 °C, the plates were incubated with serial dilutions of IL-8 samples and standards. After incubation with the detection antibody and the tetramethylbenzidine substrate, absorbance was measured at 405 nm using an ELISA reader. The assay detection limit was 3.1 pg/ml IL-8.

**Isolation of Cellular Fractions**—For nuclear protein isolation, cells were scraped into ice-cold PBS. The cell pellet was resuspended in lysis buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40, and protease inhibitor mixture (Sigma-Aldrich); incubated for 10 min on ice; and centrifuged. The supernatant (cytosolic fraction) was collected, whereas the pellet was resuspended in the buffer containing 20 mM HEPES, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 25% glycerol, and a protease inhibitor mixture (Sigma-Aldrich). After incubating for 10 min on ice, the samples were centrifuged, and the supernatants (nuclear proteins) were collected, aliquoted, and stored at −80 °C before analysis.

**Isolation of Nuclear Extracts and Immunoprecipitation Assay**—After isolation of nuclear fraction using the methods previously mentioned, mouse monoclonal anti-FLAG was added to the supernatant of the nuclear extracts, and the mixture was rotated overnight at 4 °C. Protein G PLUS-agarose (30 μl; Santa Cruz Biotechnology) was then added to the antibody-mixed nuclear extracts, after which the samples were rotated at 4 °C for 3 h. The antibody-added nuclear extracts were then washed three times, after which 6× SDS sample buffer was added.
added. Finally, immunoprecipitates were collected by centrifugation and subjected to SDS-PAGE. The nuclear (heterogeneous nuclear ribonucleoprotein) and cytoplasmic (β-actin) markers verified the identity and purity of the fractions.

Quantitation of Intracellular Cholesterol Contents—Intracellular cholesterol contents were measured according to the manufacturer’s instructions for the total cholesterol colorimetric/fluorometric assay kit (Biovision, Milpitas, CA).

Human Tissue Sampling—Human colonic tissue samples were obtained from the ulcer margins and normal surrounding areas during colonoscopy in patients with CD. Diagnosis of IBD was based on clinical history, physical examination, endoscopic appearance, histologic findings, and radiologic studies. CD was defined by 1) evidence of a granuloma in any one biopsy from upper and/or lower endoscopy; 2) the absence of a granuloma, the presence of skip lesions on colonoscopy, and/or the presence of mucosal abnormality with microscopic focal chronic inflammatory changes in upper endoscopy or segmental small intestinal radiologic findings consistent with CD; 3) the presence of perianal disease (i.e. abscesses, fistulae, and large skin tags); and/or 4) the presence of transmural inflammation, such as stricturing or fistulizing disease. Intestinal mucosal specimens were obtained from colonicoscopic biopsies. At least two biopsies were taken from the most inflamed area, and if inflammation was not evident during colonoscopy, biopsies were taken from a normal appearing area. Inflamed or non-inflamed status of all intestinal biopsies was macroscopically assessed during endoscopy. Informed written consent was obtained from the guardians before the biopsies, and a brief gastrointestinal history was taken. Written informed consent to acquire biopsy specimens was obtained from patient guardians. The study was carried out in accordance with the Helsinki Declaration after approval was received from the institutional review board of Yangsan Pusan National University Hospital (approval number 05-2012-012).

Colitis Induction in Mice—The mice were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate for 7 days. All mice were individually housed in ventilated cages with free access to feed and water and acclimated to standard laboratory conditions (12-h light/dark cycle, temperature 22 ± 1 °C). This animal study was approved by the Pusan National University institutional animal care and use committee (approval number PNU-2015-0786). Six-week-old female C57BL/6 mice weighed an average of 16–18 g. To induce large intestinal injury, mice were administered ad libitum with DSS (M, 36,000–50,000; MP Biomedical, Solon, OH) in drinking water at 3% w/v for 5 days, which was then replaced with regular drinking water for another 5 days before sacrifice. The isolated intestine was rolled in the “Swiss roll” formation and fixed in 4% paraformaldehyde solution. HistoQuest software (TissueGnostics, Vienna, Austria).

Confocal Microscopy—Cells were incubated in a glass bottom culture dish. HCT-8 cells were treated with vehicle or 1 mM MβCD for 4 h. The process of confocal microscopy was described in a previous report (50).

ChIP Assay—Procedures for the ChIP assay were described in a previous report (50). The 5’ forward and 3’ reverse complement PCR primers for amplification of each gene were as follows: human EGR-1 promoter, 5’-CTA GGG TGC AGG ATG GAG GT-3’ and 5’-GAA CAC TGA GAA CGG TGC AG-3’; human PPARγ promoter, 5’-TCA TGT AGG TAA GAC TGT GTA G-3’ and 5’-CGT TAA AGG CTG ACT CTC GT-3’.

RNA Immunoprecipitation—Immunoprecipitation of protein-RNA complexes was performed using a modified protocol of chromatin IP (52). The cytoplasmic extract was incubated overnight at 4 °C with 5 mg of either goat anti-mouse IgG (non-specific control) or anti-HuR Ab. RNA was then extracted with TRizol reagent and subjected to real-time PCR.

Isolation of Mouse Intestinal Epithelial Cells—Animal care and experimental procedures were conducted in accordance with the university committee’s guidelines for animal care and use. Isolation of mouse intestinal epithelial cells was described previously (50).

Statistical Analysis—Data were analyzed using SigmaPlot 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, analysis of variance was performed. Data that did not meet normality assumptions were subjected to Kruskal-Wallis analysis of variance based on ranks, and then pairwise comparisons were made using the Student-Newman-Keuls method.

Author Contributions—Project design and hypotheses were made by Y. M. and S.-H. P. S.-H. P., J. K., and M. Y. conducted experiments and analyzed data. J.-H. P. and Y. S. K. performed clinical sampling and assessment. Y. M. and S.-H. P. prepared the manuscript. Y. M. supervised the overall project.

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