ER stress and the unfolded protein response in intestinal inflammation

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McGuckin MA, Eri RD, Das I, Lourie R, Florin TH. ER stress and the unfolded protein response in intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 298: G820–G832, 2010. First published March 25, 2010; doi:10.1152/ajpgi.00063.2010.—Endoplasmic reticulum (ER) stress is a phenomenon that occurs when excessive protein misfolding occurs during biosynthesis. ER stress triggers a series of signaling and transcriptional events known as the unfolded protein response (UPR). The UPR attempts to restore homeostasis in the ER but if unsuccessful can trigger apoptosis in the stressed cells and local inflammation. Intestinal secretory cells are susceptible to ER stress because they produce large amounts of complex proteins, for secretion, most of which are involved in mucosal defense. This review focuses on ER stress in intestinal secretory cells and describes how increased protein misfolding could occur in these cells, the process of degradation of misfolded proteins, the major molecular elements of the UPR pathway, and links between the UPR and inflammation. Evidence is reviewed from mouse models and human inflammatory bowel diseases that ties ER stress and activation of the UPR with intestinal inflammation, and possible therapeutic approaches to ameliorate ER stress are discussed.

endoplasmic reticulum; protein misfolding; goblet cell; Paneth cell; inflammatory bowel diseases

THE ENDOPLASMIC RETICULUM (ER) is responsible for synthesis of polypeptides and posttranslational modification and folding of peptides to form functional proteins for cellular function or secretion. ER stress occurs when there is an excess of misfolded proteins and has been linked with an increasing number and wide variety of inherited and sporadic human diseases including neurodegenerative diseases, developmental disorders, cancer, diabetes, cystic fibrosis, and infectious and inflammatory disease (30, 55, 88, 92, 99). The purpose of this review is to highlight the particular relevance of ER stress and its downstream influences to intestinal physiology and pathology. Emphasis will be given to epithelial secretory cells that manufacture large amounts of complex proteins in the ER and therefore are prone to protein misfolding and hence are likely to have highly developed tightly regulated mechanisms for dealing with ER stress.

Initially the review introduces ER function, ER stress, and the consequent unfolded protein response (UPR) and ER-associated protein degradation (ERAD) in sufficient detail to introduce the reader to the major concepts and players involved in these complex intracellular pathways. It should be noted that there are recent, more comprehensive reviews on each of these aspects that can be accessed for further detail (52, 90, 119, 139). The review then discusses the influence of the UPR on the apoptosis, inflammation and autophagy pathways, and how external factors can modulate the UPR. The challenges facing the ER and UPR in intestinal secretory cells and emerging evidence from animal models implicating ER stress in secretory cells with intestinal inflammation are described. Evidence for ER stress in human intestinal inflammation is then presented and the possible therapeutic targeting of this pathway in inflammatory bowel diseases (IBD) discussed. The review concludes by identifying the major research challenges that need to be explored to gain a better understanding of this aspect of gastrointestinal physiology and pathology.

Introduction to ER Stress and the UPR

ER function. Although polypeptide synthesis can occur in free ribosomes in the cytoplasm, the cisternal network of membranes with attached ribosomes known as the rough ER is a major site for protein synthesis in eukaryotic cells and the site of synthesis of virtually all secreted and membrane-integrated proteins. Protein translation occurs within the ribosomes, and the nascent polypeptide chains emerge in the ER lumen where posttranslational modifications occur (including N-glycosylation, and intra- and intermolecular disulfide bond formation); the polypeptides are folded into specific tertiary structures essential to protein function. Although many of these processes are determined by the amino acid sequence of the protein, a range of molecules resident within the ER, including chaperones and enzymes, are essential for appropriate biosynthesis and correct folding. Key chaperones include the heat shock proteins [e.g., glucose-regulating peptide (GRP)78, GRP94] and glycoprotein-associated chaperones (e.g., calnexin and calreticulin). Generally these chaperones disengage from pro-
tein once the correct conformation is achieved, and therefore they accumulate with misfolded proteins. Chaperone production is upregulated in response to increased misfolding, and measurement of increased chaperones, particularly GRP78, is commonly used as a marker of ER stress (76). Enzymes such as the glycosyltransferases and glycosidases, which catalyze N-glycan formation and trimming, and the protein disulfide isomerases, which both build and reduce disulfide bonds, can be critical for correct protein folding. In fact, the most commonly deployed experimental induction of ER stress is the use of the microbial toxin tunicamycin, which blocks N-glycosylation, thereby causing misfolding of many proteins in the ER. The ER is also the most important intracellular store of Ca\(^{2+}\) with the result that disturbances to intracellular Ca\(^{2+}\) also cause protein misfolding and ER stress. This can be achieved experimentally in cultured cells with the sarco/ER Ca\(^{2+}\) ATPase inhibitor thapsigargin.

**ER stress.** The consensus opinion is that a small proportion of all proteins will misfold because this is a highly complex and somewhat stochastic process and that the proportion of misfolding is likely to increase with increasing protein complexity. Thus, as part of routine housekeeping, the ER has highly developed mechanisms to recognize misfolded proteins and remove them for degradation (139). In addition to inhibiting N-glycosylation and alterations of ER Ca\(^{2+}\) concentrations, factors that can lead to increased misfolding include increased rate of protein synthesis, missense polymorphisms in individual proteins, energy (ATP) depletion, osmotic stress, viral infection, and increased temperature. When protein misfolding exceeds the background threshold and ER homeostasis is not maintained, the condition known as ER stress is reached, which engages a network of signaling and transcriptional events collectively known as the UPR. Depending on the nature of the stressor, ER stress is often transient and well controlled by appropriate UPR function; however, prolonged ER stress can have major implications for the protein production capabilities of the cell, affect other cellular functions, and ultimately may result in premature apoptosis. Nevertheless, it is important to recognize that cells producing large amounts of protein are highly likely to experience low-level background ER stress and switch on elements of the UPR as part of their normal cellular physiology, and to consequently have specific adaptions, as discussed later for intestinal secretory cells.

**Coping with ER Stress: the UPR**

**Elements of the UPR.** The UPR involves a series of enzymes and transcription factors typically engaged in a parallel and multifactorial manner to restore ER homeostasis, as depicted schematically in Fig. 1. In fact, a key element of the trigger for virtually all UPR pathways is the ER chaperone GRP78 (product of the HSPA5 gene, also known as BiP), which associates with the key ER-resident UPR pathway initiating molecules inositol-requiring enzyme (IRE)1-\(\alpha/\beta\), protein kinase RNA-like ER kinase (PERK), and activating transcription factor (ATF)6-\(\alpha/\beta\). As discussed above, GRP78 accumulates with misfolded proteins, and the sequestration of GRP78 from the UPR initiating molecules is an important component in their activation and induction of the UPR.

IRE1, with two forms, \(\alpha\) and \(\beta\), encoded by distinct genes with different expression patterns, is a ribonuclease that is activated by autophosphorylation following disengagement from GRP78 and/or direct recognition of misfolded protein complexes. The major substrate for the IRE1 ribonuclease is the mRNA encoding the X box-binding protein 1 (XBP1) transcription factor. IRE1 splices the XBP1 mRNA, resulting in the coding of a transcription factor that induces UPR target genes, including chaperones, protein disulfide isomerases (PDIs), and components of ERAD (78, 151). Conversely, the unspliced XBP1 mRNA encodes a transcription factor that actively represses UPR target genes (152). Hence the ratio of spliced to unspliced XBP1 mRNA is a useful measure of UPR activation. More recently, IRE1 has been shown to also degrade ER-localized mRNAs in a distinct role associated with ER stress-induced apoptosis (44).

PERK shares structural similarities with IRE1 and is also activated by autophosphorylation following disengagement from GRP78 and/or sensing of misfolded proteins. The primary function of PERK is to phosphorylate the eukaryotic initiation factor-2; (eIF2-\(\alpha\)) component of the translation initiation complex, resulting in arrested protein translation, thereby reducing the load entering the ER (46, 47). However, PERK phosphorylation of eIF2-\(\alpha\) also influences transcription via ATF4 transcription factor, somewhat paradoxically, promoting the production of specific ER-resident proteins important for restoration of ER homeostasis.

ATF6 (two forms, \(\alpha\) and \(\beta\), which can heterodimerize and are encoded by distinct genes) moves after disengagement from GRP78 and/or sensing of misfolded proteins from the ER to the Golgi apparatus, where it is cleaved by the sphingosine-1-phosphate and sphingosine-2-phosphate Golgi resident proteases. This releases into the cytoplasm the active ATF6 transcription factor, which moves to the nucleus to modulate gene expression (49). Although these three pathways have reasonably distinct downstream functions, they are engaged in a coordinated fashion and act in concert, and much is yet to be understood about interactions between the pathways and the integration of their downstream effectors to restore ER homeostasis (118).

**Functional consequences of the UPR.** There are three main functional outcomes effected by the UPR: 1) decreased translation, 2) restoration of protein folding, and 3) degradation of misfolded proteins (ERAD). The PERK-initiated inhibition of translation discussed above appears to be an attempt to limit further misfolding by inhibiting formation of major protein products while somehow permitting synthesis in the ER of protein products of the UPR target genes. Downstream targets of the UPR include genes encoding many ER resident chaperones, PDI, and ERAD components. In addition, the size of the ER can be increased by the UPR, which increases the capacity of the cell to make correctly folded proteins as well as deal efficiently with terminally misfolded proteins (15).

Degradation of misfolded proteins, or ERAD, is itself a complex and highly regulated process because protein degradation cannot happen within the ER but rather occurs via the 26S proteasome in the cytoplasm (139). The challenge for ERAD is to recognize misfolded proteins in the ER and then to prepare them for removal via an incompletely defined ER membrane retrotranslocation channel associated with the SEC61 complex and derlin proteins (83, 130). Misfolded proteins must first be recognized as such, possibly involving recognition of inappropriate exposure of hydrophobic patches.
and associated chaperones such as GRP78. Recognition of appropriate termination of N-glycosylation appears to also be an important component in permitting normal exit from the ER. Misfolded proteins can have their N-glycans modified by addition of a glucose catalyzed by the UDP-glucose:glycoprotein glucosyltransferase, resulting in the misfolded proteins being returned to the calreticulin/calnexin cycle (16–18). Appropriate intermolecular disulfide bond formation is critical to correct folding, and conversely reduction of these disulfide bonds is required before misfolded proteins can be removed from the ER. A family of PDIs, likely to have substrate specificity, are responsible for both formation and reduction of disulfide bonds (48). The importance of the secretory cell PDI, anterior gradient 2 (AGR2) (110), which we have found to be highly upregulated during ER stress in intestinal cells in vitro and in vivo (unpublished observations), will be emphasized later in the review. Delivery of misfolded proteins to the retrotranslocation channel differs for soluble and membrane-spanning misfolded proteins. Removal of membrane-associated proteins appears to involve cytoplasmic heat shock proteins, which complex with ubiquitin E3 ligase, resulting in removal of the protein from the ER membrane and subsequent degradation by the proteosome (96). In contrast, for soluble proteins (most of which will be N-glycosylated) delivery to the retrotranslocon involves N-glycan-binding lectins including ER degradation-enhancing α-mannosidase-like lectins (EDEM)1, EDEM2, and EDEM3 (106), osteosarcoma amplified 9 ER lectin (OS9) (23), and XPTB3 (56). This is a dynamic, highly regulated, and probably competitive process. Thus the complex series of events required for removal of misfolded proteins and the requirement for removal through a membrane translocon suggests that large glycoproteins, such as the mucin glycopro-
proteins discussed later, would represent a substantial and possibly insurmountable challenge for ERAD. However, there have been suggestions that an alternative possibility for large aggregates could be removal via formation of lipid droplets formed from ER membrane (114) or budding of ER (156). Thus the UPR regulates a complex series of events promoting successful protein biosynthesis and maintenance of general ER function and capacity.

Interaction of the UPR with other pathways. In addition to affecting events in the ER, the UPR can have wider influences on cell biology with important ramifications for intestinal epithelial cells.

APOPTOSIS. Prolonged or severe ER stress can result in premature apoptosis via the intrinsic or Fas-mediated pathways (72, 119). The classical intrinsic pathway of apoptotic cell death involves an altered balance between apoptotic inhibitors (Bcl2 family) and proapoptotic proteins (Bax, Bak), leading to cytochrome c release from mitochondria, caspase activation, and ensuing intracellular controlled degradation (42). Although multiple components of the UPR have been demonstrated to modulate apoptosis both in vitro and in vivo, we have an incomplete understanding of how the various pro- and anti-apoptotic signals are integrated. Perhaps the most important proapoptotic pathway involves PERK activation-triggered ATF4-mediated production of the transcription factor C/ enhancer binding protein (EBP) homologous protein (CHOP or GADD153). CHOP has been shown to induce apoptosis via increasing transcription of the proapoptotic gene Bim, decreasing transcription of the antiapoptotic gene Bcl2 (115), and via production of the ER resident oxidase, ERO1-α, which sensitizes 1,4,5-triphosphate (IP3) receptor to IP3, thus triggering Ca2+ -mediated apoptosis. ER stress also results in dephosphorylation of Bim by protein phosphatase 2A, thus preventing Bim ubiquitination and degradation, and Bim appears a critical component of ER stress-induced apoptosis in a variety of cell types (115). ATF6-α directly increases CHOP transcription in myeloblasts, thereby having a similar effect to ATF4 (95). IRE1 activation has been shown to trigger phosphorylation of JNK via TNF receptor-associated factor (TRAF2) and apoptosis signaling kinase 2 (ASK2), leading to increased apoptosis possibly involving direct activation of caspase 12 by TRAF2 (70, 136, 149). IRE1 and TRAF2 can also complex with IKK and subsequently modulate NF-κB regulation of apoptosis (59). IRE1-α also interacts directly with proapoptotic Bak and Bax (85), which like many apoptosis proteins are found within the ER, and itself can be inhibited by Bax inhibitor-1, which can therefore modulate ER stress-induced apoptosis (86). A model has been proposed where the relative balance of IRE1, ATF6, and PERK signaling alters temporally in prolonged ER stress, pushing the balance toward proapoptotic signals (84).

Release of Ca2+ from the ER into the cytoplasm following ER stress has also been linked with both the intrinsic and Fas-mediated pathways of apoptosis via calcium/calmodulin-dependent protein kinase II-γ (CaMKII-γ) and JNK, with multiple cell types in CAMKII-γ−/− mice more resistant to ER stress-induced apoptosis in vivo (134). The apoptotic response by the cell is dependent on both cellular differentiation and the context of the stress. This is because the regulation of ER stress-induced apoptosis involves not only the severity, nature and duration of the stressor, and the activation of the various UPR elements, but also expression of critical pro- and anti-apoptotic proteins by specific cell types.

INFLAMMATION. There are direct links between ER stress/ UPR and both local and systemic inflammation, which suggest that ER stress itself promotes inflammation, a pathway that is likely to have evolved because viral infection is an important common ER stressor. NF-κB activation is considered central to intestinal inflammation (1, 117) and has been observed in unaffected tissues from patients with inflammatory bowel disease and in their asymptomatic identical twins (10). Importantly, ER stress triggers NF-κB activation via at least four direct pathways: 1) IRE1-α complexing with IKK via the adaptor protein TRAF2, leading to degradation of Iκ-Box (59), 2) PERK-initiated blockage of Iκ-Box translation (29), 3) a direct interaction between cytoplasmic GRP78 and the IKK complex (126), and 4) disturbance in Ca2+ metabolism and production of reactive oxygen species (108). ER stress results in local hyaluronan deposition by cultured colonic smooth muscle cells, thus promoting leukocyte recruitment to the vicinity of stressed cells (89). ER stress in the liver results in activation of the cyclic-AMP-responsive-element-binding protein H transcription factor via an ER to Golgi protease-mediated mechanism similar to that for ATF6, resulting in systemic release of acute inflammatory factors (153). Thus in combination with other local and systemic factors, ER stress may be an important component of some types of chronic inflammation including diseases like IBD. In fact, there is evidence that ER stress in pancreatic β-cells promotes inflammation in type II diabetes (107).

AUTOPHAGY. Autophagy is a process by which intracellular debris and organelles are surrounded by a membrane to facilitate degradation by fusion with lysosomes (50). Autophagy has relevance for ER stress because it is engaged to degrade misfolded protein aggregates that cannot be removed by ERAD and because there are multiple connections between the UPR and regulation of autophagy (7). Both IRE1, via phosphorylation of JNK (103), and PERK, via phosphorylation of eIF2-α (75), can be involved in initiating autophagy in response to ER stress in different contexts. Additionally, ER stress-initiated release of Ca2+ triggers CaMKKβ, which in turn activates 5’-AMP-activated protein kinase (AMPK) (57, 58). AMPK is a major regulator of mammalian target of rapamycin (mTOR), which is the master regulator of autophagy in mammalian cells (50). However, increased cytosolic Ca2+ also induces phosphorylation of protein kinase Cδ (PKCδ), which induces autophagy in an mTOR-independent manner (121, 122). Thus ER stress will often be accompanied by induction of autophagy, and defects in components of either process in intestinal cells are likely to affect the other pathway.

Although the UPR is primarily driven by events within the ER, a variety of cellular and extracellular factors can influence UPR signaling rather than modulating ER stress itself. In an example relevant to the intestinal microenvironment, Toll-like receptor signaling has recently been shown to suppress ER stress-induced production of CHOP by inhibiting eIF2-α induction of ATF4 (146). Also relevant to intestinal inflammation, the tolerizing cytokine IL-10 has been shown to affect one arm of the UPR by a p38-mediated inhibition of the nuclear translocation of the ATF6 transcription factor (126). Conversely, the inflammatory cytokine TNF-α induces intracellular production of reactive oxygen species, which in turn induce
ER stress (148). Infection by many common viruses, including enteroviruses, leads to ER stress attributable to the production of viral proteins (13, 22, 61, 62, 65, 68, 99, 112, 129). Although ER stress in virally infected cells can cause ER stress-mediated apoptosis (68, 69, 92, 99), there are numerous examples of manipulation of the ER stress and UPR by viral proteins to the advantage of the virus (5, 19, 22, 62, 65, 112).

Another consideration is that viral infection may combine with either environmental ER stressors or a genetic predisposition to misfolding to cause ER stress. A good example of this occurs in individuals with familial and sporadic interstitial lung disease that is linked with misfolding mutations in the SFTPC gene, which encodes the surfactant protein SP-C expressed by respiratory epithelial cells. Although these mutations cause misfolding of SP-C and activation of the UPR and NF-κB activation, the ER stress is markedly increased by respiratory syncytial virus infection of respiratory cells, leading to greatly increased apoptosis (13). This nuance is thought to underlie the variability of age of onset of lung disease in pedigrees with these mutations. Thus, more generally, the local microenvironment including infection, cellular differentiation/gene expression, and the nature of the ER stressor will all contribute to the tenor of the consequent UPR.

**Intestinal Secretory Cells: Major Challenge for the ER**

Secretory cells that manufacture large amounts of protein for export naturally present a major challenge for the ER. In addition to the above-mentioned example of ER stress occurring in pancreatic β-cells, which manufacture large amounts of insulin, ER stress occurs in plasma cells, which secrete large amounts of antibody (15, 63, 90, 128). It is highly likely that secretory cells, as an integral part of their phenotype, have highly developed mechanisms to maximize correct folding of proteins and to deal with the misfolding that will inevitably occur. The intestine contains secretory cells that manufacture very large amounts of complex proteins for secretion. The major examples of secretory cells in the intestine are the goblet cells that produce the mucus barrier and the Paneth cells that produce a range of antimicrobial molecules.

Goblet cells are found throughout the intestinal tract from the duodenum to the rectum (see Fig. 2). Their major synthesized proteins are the secreted mucin glycoproteins, which, because of their large size (>5,000 amino acids), high disulfide content, and homo-oligomerization, are likely to present a major challenge to achieve correct folding. In the duodenum the goblet cells produce the MUC2 and MUC5AC mucins, whereas in the remainder of the intestine in health the goblet cells produce mainly MUC2 (20) and some MUC6 (25). However, MUC5AC can be expressed in some human colonic crypts (9) and can be upregulated during inflammation (37, 124). The secreted mucins contain large extended central proline, threonine, and serine-rich (PTS) domains, which are O-glycosylated in the Golgi after transfer from the ER. Before transfer to the Golgi, the NH2- and COOH-terminal cysteine-rich domains and additional “cys-domains” that are interspersed in the PTS domains are N-glycosylated, folded (with formation of numerous disulfide bonds), and then dimerized via disulfide linkages in the COOH termini (2). Following O-glycosylation in the Golgi, the mucin dimers are then oligomerized further via the NH2 terminus and packaged into granules for secretion (40, 133). Collections of stored mucin secretory granules form the thecal structure, which is the morphologically defining characteristic of goblet cells. However, these granules are constitutively secreted at a high rate. Pulse chase studies in the mouse indicate a turnover of less than 5 h, thus requiring a continuous high level of mucin biosynthesis. Following secretion, mucin granules expand 100-
to 1,000-fold upon hydration (140) to form the major macromolecular component of the secreted mucus barrier, which separates the luminal microbial flora from the intestinal epithelium (66). Although mucins are the major secreted product of goblet cells, it should be stressed that goblet cells produce a range of other molecules that are likely to be important functional components of the mucus barrier, including trefoil peptides, RELMβ, and FCGBP (67). It has recently emerged that the transcription factor, S-adenosyl-L-methionine-pointed domain E-twenty-six family transcription factor (SPDEF), is a critical inducer of the goblet cell phenotype in mucosal tissues, including the intestine (21, 100, 109). SPDEF induces expression of multiple genes including the mucins and enzymes involved in mucin glycosylation. One key gene induced with the mucins encodes an ER resident PDI, AGR2, which is likely to be critical for correct disulfide bond formation in mucin glycoproteins (21, 100, 110). More broadly, SPDEF is likely to promote expression of genes required for appropriate ER biosynthesis of mucins and to provide appropriate modulation of the UPR for these protein-manufacturing cells.

Paneth cells are normally found in the base of the crypts of the small intestine, but Paneth cell metaplasia in the colon can occur with chronic inflammation in Crohn’s disease (CD) and ulcerative colitis (UC) (see Fig. 2). Paneth cells manufacture a range of proteins for secretion including defensins, lysozyme, antimicrobial lectins, and collectins (145). The small defensin peptides are cysteine rich, and disulfide bonds are important for correct folding (132). Paneth cells also produce MUC2, though in much smaller amounts than goblet cells, which ensures that the antimicrobial molecules are secreted in a milieu of mucus in the crypt. Like the goblet cells, Paneth cells store the secreted products in granules and constitutively secrete these proteins, thus necessitating continuous biosynthesis, and the PDI, AGR2, is also highly expressed by Paneth cells (154).

Although goblet cells and Paneth cells appear to cope with the high levels of baseline protein biosynthesis, environmental and inflammatory triggers promote increased synthesis and secretion by these cells. Inflammatory cytokines (35, 64, 73, 81, 87), microbial molecules recognized by Toll-like receptors and nucleotide-binding oligomerization domain (NOD) proteins (31, 80, 82), growth factors (113), lipid mediators (39), and hormones (34, 54) increase MUC2 transcription. Secretion of mucins by goblet cells is increased by pAMPs, toxins, and nervous stimuli (35, 36, 73, 91, 125). Production and secretion of defensins, lectins, and other Paneth cell products are also increased by bacterial and viral products and inflammatory cytokines (5, 6, 74, 102, 105, 120, 141). These conditions are likely to place stress on the ER to maintain homeostasis and appropriate UPR and ERAD function simply due to the increased protein biosynthesis. Compounding this stress, environmental factors such as viral infection, plant and microbial toxins (79), and inflammatory factors such as TNF-α and reactive oxygen species (148) can provide direct stress on the ER. Thus, although an individual carrying polymorphisms in a secreted protein may have apparently normal secretory cell function in the basal state, challenges from ingested toxins or bacterial or viral infection could result in severe secretory cell ER stress (as was discussed earlier for the SP-C protein and viral infection in interstitial lung disease, Ref. 13).

Evidence linking ER stress with intestinal inflammation. Emerging evidence from mouse models, together with the phenotype of (human) IBD, and IBD genetic data, are collectively pointing toward the importance of the ER stress and related pathways in intestinal inflammation (see Fig. 3). We propose that this should be considered as a pathway, with multiple levels at which environmental and genetic factors could combine to promote unresolved intestinal inflammation. Because intestinal secretory cells produce important elements of the mucosal barrier known to protect from mucosal infection and inflammation (123, 137), ER stress in these cells is likely to promote inflammation in two ways: 1) by reducing the effectiveness of the mucosal barrier attributable to decreased

Fig. 3. Pathways that could lead to ER stress in human inflammatory bowel diseases (IBD). Examples are shown of genes in these pathways linked with intestinal inflammation in mouse models or with IBD in genetic studies. MDR1, multidrug resistance protein 1; SNP, single nucleotide polymorphism; MUC, mucin; AGR2, anterior gradient 2; ROS, reactive oxygen species; TNFR, tumor necrosis factor receptor; IRF, interferon regulatory factor.
secretion of antimicrobial molecules and mucus, and premature apoptosis, and 2) by UPR-initiated inflammatory signals released by the stressed secretory cells.

Evidence from animal models. Data from several murine models unequivocally demonstrate how defects in ER stress-related pathways lead to intestinal inflammation either by promoting protein misfolding or by inappropriate UPR responses to normal levels of misfolding (summarized in Table 1). We have described two strains of mice with spontaneous intestinal inflammation that were independently generated by two experimental ethylNitosourea (ENU) mutagenesis programs, both of which involve missense mutations in Muc2, leading to misfolding in the ER (51). In Winnie and Eeyore mice, although goblet cells initially produce and secrete Muc2, a proportion of Muc2 precursor progressively accumulates within ER vacuoles, the size of the theca containing mature mucin for secretion diminishes, and these cells reach a point where further Muc2 biosynthesis appears to cease (see Fig. 2). Similarly, Paneth cells, which also synthesize Muc2, show vacuolation and reduced numbers of granules for secretion (see Fig. 2). The ER stress triggers multiple elements of the UPR, including splicing of the Xbp1 mRNA and upregulation of Grp78, Agr2, Atf4, and Chop. Winnie and Eeyore mice progressively develop spontaneous colonic (and to a lesser extent distal ileal) inflammation with increased local production of inflammatory cytokines, increased density, activation of antigen-presenting cells, and a consequent dominant TH17 T cell response akin to that found in human UC (R. D. Eri, R. J. Adams, T. V. Tran, I. Das, D. K. Roche, I. Oancea, C. W. Png, P. L. Jeffery, G. L. Radford-Smith, M. C. Cook, T. H. Florin, and M. A. McGuckin, unpublished observations). It is likely in this model that both mucus depletion and ER stress signaling contribute to the development of inflammation.

Similarly, mice deficient in the Agr2 PDI, which is coexpressed with Muc2 in goblet cells and appears to bind to Muc2 oligomerization domains, also develop spontaneous intestinal inflammation (110, 154). However, rather than germ line loss of Agr2 producing a phenotype like Winnie and Eeyore with misfolded Muc2 within goblet cells, germ line deficiency in this PDI results in an almost complete loss of Muc2 production by goblet cells, accompanied by only relatively mild ER stress (110). This suggests that, with absence of Agr2 in Agr2 germ line knockouts, differentiating goblet cells shut down Muc2 production, possibly by a UPR-mediated mechanism (110, 154). In contrast, Zhao et al. (154) also reported an expansion of Paneth cells together with an ectopic location of some Paneth cells in the intestinal villi (154) of their Agr2 germ line knockout on a different mouse background. When this group knocked out Agr2 in an inducible fashion, a quite different phenotype emerged in mature secretory cells with the cells appearing to experience catastrophic stress, leading to major epithelial damage (154). Both the inducible and germ line Agr2 knockouts developed both terminal ileitis and colitis, with inflammation developing within several days of loss of Agr2 in the inducible model (154). Another example of misfolding leading to intestinal inflammation is in human HLA-B27-transgenic rats. Human HLA-B27 appears to misfold in rat macrophages, and perhaps other cells, which is accompanied by inflammation in the joints and in the intestine (26, 135). In addition to increased misfolding being a pathway to inflammation, inappropriate UPR responses to the normal levels of misfolding can also initiate inflammation.

The first evidence for a link between inappropriate UPR and intestinal inflammation was knockout of Ire1-β, which is the isoform of Ire1 expressed in colonic and gastric epithelial cells (8). Deficiency in Ire1-β induced a threefold increase in Grp78, indicative of increased ER stress. Ire1-β knockout mice are not reported to develop spontaneous intestinal inflammation but do show increased sensitivity to dextran sodium sulfate (DSS)-induced colitis (8). Interestingly, in these studies DSS was shown to induce ER stress in Chinese hamster ovarian cells in vitro, implicating the ER stress pathway as part of the complex

Table 1. Murine genetic models of defective protein folding, UPR, and autophagy

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<tr>
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<th>Genotype and Phenoty</th>
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<td><strong>Defects in biosynthesis and folding</strong></td>
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<td>Winnie &amp; Eeyore/Muc2</td>
<td>Genotype–SNP missense mutations in D3 and D4 domains of the Muc2 mucin glycoprotein.</td>
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<td>ENU mutants</td>
<td>Phenotype–Goblet cell and Paneth cell ER stress, Muc2 precursor accumulation, UPR activation, spontaneous TH17-mediated inflammation, and increased sensitivity to DSS.</td>
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<td>Agr2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Genotype–Knockout of Agr2 ER protein disulfide isomerize coexpressed with Muc2 in goblet cells and Paneth cells.</td>
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<td>Phenotype–ER stress, abrogation of Muc2 biosynthesis, spontaneous intestinal inflammation. Inducible knockout causes Paneth cell and goblet cell apoptosis and severe inflammation.</td>
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<td>Xbp1&lt;sup&gt;−/−&lt;/sup&gt;ΔΔCre</td>
<td>Genotype–Intestinal specific inducible knockout of the Xbp1 UPR transcription factor activated by Ire1-β.</td>
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<td>Phenotype–Paneth cell and goblet cell apoptosis, spontaneous ileal inflammation, increased sensitivity to DSS colitis.</td>
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<td>Woodrat/Mbtps1</td>
<td>Genotype–SNP missense mutation in Golgi enzyme encoded by Mbtps1 that activates Atf6.</td>
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<td>ENU mutant</td>
<td>Phenotype–Spontaneous mild intestinal inflammation, increased sensitivity to DSS colitis.</td>
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<td>Ire1&lt;sup&gt;β−/−&lt;/sup&gt;</td>
<td>Genotype–Knockout of Ire1-β UPR endonuclease which cleaves Xbp1 mRNA.</td>
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<td>Genotype–Hypomorphic for autophagy proteins Atg5 and Atg16L1 in intestine.</td>
<td>14</td>
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<td>Phenotype–Paneth cell pathology, decreased production of secretory granules, and vacuolation of the ER.</td>
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SNP, single nucleotide polymorphism; ENU, ethylNitosourea; ER, endoplasmic reticulum; UPR, unfolded protein response; DSS, dextran sodium sulfate; Ire, inositol-requiring enzyme; Chop, C/EBP homologous protein.
etiology of this commonly used model of colitis. More recently, deficiency of the Xbp1 transcription factor in intestinal epithelial cells has been shown to induce pathology in both Paneth and goblet cells in the small intestine and spontaneous ileitis (71). Xbp1-deficient intestinal tissue showed evidence of ER stress including increased Grp78, Atp4, and Chop, and moreover overactivation of the Ire1 exonulease also accompanied deficiency of its substrate Xbp1 mRNA. Importantly, even a proportion of mice haploinsufficient for Xbp1 developed spontaneous ileitis, suggesting that the amount of Xbp1 is critical for normal homeostasis in intestinal secretory cells. This villin promoter-driven intestinal specific knockout also induces partial Xbp1 deficiency in the colon, which leads to some colonic goblet cell depletion, and, although these mice are not reported to develop spontaneous colitis, they are more sensitive to DSS (71). These results demonstrate the importance of Xbp1 for ER homeostasis in intestinal secretory cells, as had previously been demonstrated in other cells in vitro (15). In another example of defective UPR responses leading to intestinal inflammation, Woodrat mice with an ENU-induced missense hypomorphic mutation in Mbpts1, one of the Golgi resident enzymes responsible for cleaving ATF6 develop mild spontaneous colitis but not ileitis (12). These mice have mildly elevated production of inflammatory cytokines by the distal colon and an enhanced sensitivity to DSS-induced colitis. This mutant retains some enzymatic function as null animals die in utero. In contrast to the Ire1-β and Xbp1 deficiency models, Woodrat mice do not show increased expression of ER stress chaperones. In fact, these mice show decreased expression of Grp78 and Grp94 following induction of ER stress with DSS. Woodrat mice also show decreased survival in response to systemic challenge with tunicamycin, highlighting the importance of appropriate ATF6-regulated gene expression in coping with ER stress (12). Together these murine models demonstrate that aberrations in different elements in the UPR can result in related but distinct intestinal inflammatory phenotypes. On the other hand, some elements of the UPR may promote inflammation, as exemplified by deficiency of the Chop transcription factor, which leads to decreased susceptibility to DSS colitis, at least partly by restricting the amount of ER stress-induced apoptosis in intestinal epithelial cells (98).

The importance of autophagy in cells experiencing ER stress has been well established in nonintestinal cells in vitro (7, 103, 150). Autophagy has recently been linked experimentally with intestinal secretory cell pathology and inflammation. Mice hypomorphic for either autophagy-related gene, Atg16l1 or Atg5, both of which are involved in the molecular complex responsible for autophagosome formation, develop Paneth cell pathology with morphological similarities to Paneth cells in Crohn’s ileitis and Paneth cells in the Winnie and Eeyore Muc2 mutation models (14). The affected Paneth cells in Atg16l1 hypomorphic mice show a marked decrease in production of the granules containing antimicrobial molecules, highlighting a fundamental role of autophagy in normal homeostasis of Paneth cells. Although spontaneous inflammation has not been reported in these mice, the morphological phenotype involves accumulation of ER vacuoles, suggesting that autophagy is involved in the removal of misfolded proteins that accumulate in Paneth cells.

Evidence from IBD. Morphological, biochemical, and genetic data support involvement of intestinal secretory cell pathology in both UC and CD. In CD, there has been a surge of interest in Paneth cells because of evidence for decreased defensin production in active CD (142–145); although this is somewhat controversial (127). Paneth cell expression of the gene most strongly linked to ileal CD, CARD15 (NOD2) (77, 104), and demonstration of the microbial-derived NOD2 ligand, muramyl dipeptide, increase transcription of defensin genes (141). Although ER stress in Paneth cells has not been specifically studied in CD, morphology similar to mice hypomorphic for the autophagy gene Atg16l1 and consistent with ER stress has been shown in Paneth cells of patients carrying both ATG16L1 risk alleles (14).

Whereas CD is often accompanied by goblet cell hyperplasia, UC is characterized by fewer goblet cells with smaller thecae and decreased MUC2 production (135a, 138), leading to a thinner mucus barrier. Although similar changes in goblet cells can occur during infectious colitis and some of these changes are likely to be attributable to elements of the inflammatory response, vacuolization in secretory cells suggestive of ER stress has been observed in both inflamed and noninflamed intestines of patients with UC but not CD (28, 32, 33, 41, 94, 101). Accumulation of the nonglycosylated MUC2 precursor is well documented in UC (45, 51, 53, 124), indicating that misfolding of MUC2, as occurs in Winnie and Eeyore mice (51), occurs in UC. A fundamental question that needs to be addressed is why this phenomenon occurs and whether it is a primary event leading to colitis or secondary to inflammation.

Biochemical evidence for ER stress and initiation of the UPR in CD and UC is limited, as there have been no comprehensive studies. However, the small studies in both CD and UC that have been conducted indicate that ER stress occurs in both diseases with increased expression of GRP78 and splicing of XBP1 mRNA (51, 71, 126). The recent genome-wide association studies (GWAS) conducted in CD and UC have identified linkage of common alleles from a range of genes with both of these diseases. The autophagy genes, ATG16L1 and IRGM1, have been linked by GWAS with CD but not usually UC (43, 111, 116), and ATG16L1 was linked with severe UC in an Australian case-control study (38). Although autophagy is important in ER stress as described above, autophagy is also important in handling of intracellular bacteria and antigen presentation (24). Furthermore, autophagy can be activated by NOD2, whose gene is strongly linked with ileal CD (24, 27). Although there are no other examples of common alleles linking ER stress and related pathways with IBD, it remains likely that there are rare, more highly penetrant polymorphisms causing IBD because the common alleles identified by the GWAS only explain a minor proportion of the genetic risk of IBD. For example, it remains possible that rare misfolding mutations, such as those in Winnie and Eeyore mice, in MUC2 or other major secreted products contribute to IBD. The MUC2 gene is very large and complex and has not been thoroughly analyzed in IBD other than via the common tagging single nucleotide polymorphism (SNP) approach in the GWAS. In small case-control studies, crude variable number of tandem repeat polymorphisms in the length of the central exon encoding the glycosylated domain of MUC2 did not show linkage with UC (131), but a single missense SNP in the NH2-terminal D-domain did show linkage with CD but not UC (93). Sequence-based approaches to identify rare variants of the MUC2 gene in UC are warranted given the evidence from animal...
models, the morphology of goblet cells in UC, and the accumulation of the MUC2 precursor in UC. AGR2 polymorphisms have been linked with IBD, and altered expression of AGR2 was documented in UC intestine (155), showing that this PDI involved in mucin biosynthesis is likely to be important in some IBD. With respect to the UPR, the XBP1 gene has also been linked with CD, including identification by sequencing of rare variants with impaired transcription factor activity in the disease population (71). Figure 3 summarizes potential defects involving the ER stress and UPR pathways, which could lead to intestinal inflammation on the basis of observations from animal models and human IBD.

**ER stress and the UPR as therapeutic targets in IBD.** Whether ER stress in secretory cells is a primary defect in human IBD or arises in response to inflammatory factors, amelioration of ER stress could be therapeutic, as it is likely to help restore barrier function. Due to the importance of ER stress in a range of human diseases, including cancer, new therapeutics directed at this pathway are under development (55). Drugs that promote proper protein folding or modulate the various elements of the UPR could protect intestinal secretory cells from ER stress. For example, the eIF2-α dephosphorylation inhibitor, salubrinal, has shown efficacy in protecting cells from ER stress in vitro and in vivo (11, 97). Of course, caution is required in the clinical situation because inhibition of the UPR could negatively impact the host response to infection. This is particularly possible in the situation of a viral gastroenteritis, where the normal host defense may be to turn on the UPR to prevent viral replication in intestinal secretory cells. Therapeutic inhibition of protein degradation would be predicted to have an adverse effect on ER stress, and in fact this appears to be the case. Proteasome inhibitors slow epithelial restitution following DSS colitis in mice (60). Furthermore, protease inhibitors used to treat HIV cause gastrointestinal pathology in humans and induce ER stress and increased epithelial apoptosis in mice (147). The autophagy pathway is also potentially manipulated by drugs that affect mTOR such as the inhibitor rapamycin.

To date no specific ER stress-modulating drugs have been tested in either preclinical models or IBD. In addition, almost nothing is known regarding the influence of presently used IBD drugs on the ER stress pathway. As an example, because TNF-α induces ER stress via reactive oxygen species (148), the commonly employed and highly efficacious antibodies against TNF-α are likely to diminish ER stress in the intestine, yet there are no data available either in preclinical models or human IBD regarding the influence of these drugs on ER stress.

**Measuring Intestinal ER Stress and UPR Activation**

The GRP78 chaperone is regarded as the gold standard for measuring ER stress. We have found that measurement of GRP78 (HSPA5) mRNA or protein is a reliable measure of the extent of ER stress in intestinal cells in vitro and in the intestine in mouse models of ER stress. In our hands GRP78 elevation is reliably reversed by drugs and other factors that ameliorate stress, even when genetic mutations are the underlying primary trigger for misfolding and ER stress. Although most elements of the UPR are regulated by phosphorylation or cleavage, unfortunately there are not good antibodies available for all of these proteins or their activated isoforms, and care should be taken to validate the commercially available antibodies. Nevertheless most of the genes encoding key elements of the UPR are themselves targets of the UPR, and, for example, we have found IRE1, ATF6, ATF4, and Chop mRNA reliably upregulated in total intestinal mRNA in Winnie mice. Measurement of the spliced Xbp1 mRNA is particularly useful because this mRNA isoform is created by the action of the activated IRE1 endonuclease, and thus qPCR for spliced Xbp1 mRNA directly measures UPR activation.

**Conclusions, Future Directions, and Major Unanswered Questions**

There is a convergence of evidence from animal models and human IBD implicating ER stress and activation of the UPR as an important pathway to intestinal inflammation. Comprehensive studies in human IBD are required to determine the cellular location, extent, and nature of ER stress in both CD and UC. Gene and protein expression studies will need to be combined with morphological studies in inflamed and noninflamed tissues from IBD patients on and off treatment, so that these pathways can be characterized in the different intestinal cell populations, particularly in goblet and Paneth cells, and so that the influence of inflammation and therapies can be assessed. Major unanswered questions include: 1) Is ER stress in secretory cells in IBD a primary defect attributable to genetics or environment, or a secondary response to inflammation? 2) Which intestinal environmental factors, including microbial factors, promote ER stress? 3) Are rare polymorphisms in genes encoding major secreted proteins, proteins involved in folding, and proteins involved in ERAD and the UPR associated with human IBD? 4) Do polymorphisms in autophagy genes linked with IBD affect ER stress in intestinal secretory cells? 5) Does increased misfolding of proteins in secretory cells in IBD result in increased presentation of autoantigens? 6) Can drugs that ameliorate ER stress restore intestinal secretory cell function and dampen inflammation in animal models and human IBD? 7) Do the drugs presently used successfully to treat IBD impact the ER stress and UPR pathways?

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**DISCLOSURES**

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Review

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