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ADVANCES IN TRANSLATIONAL SCIENCE

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Intestinal Permeability and Its Regulation by Zonulin: Diagnostic and Therapeutic Implications

ALESSIO FASANO

Mucosal Biology Research Center and Center for Celiac Research, University of Maryland School of Medicine, Baltimore, Maryland

One of the most important and overlooked functions of the gastrointestinal tract is to provide a dynamic barrier to tightly controlled antigen trafficking through both the transcellular and paracellular pathways. Intercellular tight junctions (TJ) are the key structures regulating paracellular trafficking of macromolecules. Although steady progress has been made in understanding TJ ultrastructure, relatively little is known about their pathophysiological regulation. Our discovery of zonulin, the only known physiological modulator of intercellular TJ described so far, increased understanding of the intricate mechanisms that regulate gut permeability and led us to appreciate that its up-regulation in genetically susceptible individuals may lead to immune-mediated diseases. This information has translational implications, because the zonulin pathway is currently exploited to develop both diagnostic and therapeutic applications pertinent to a variety of immune-mediated diseases.

Keywords: Autoimmune Disease; Bacterial Overgrowth; Gluten; Gut Inflammation; Obesity.

Technological Primer

Recent studies indicate that besides water and salt homeostasis and digestion and absorption of nutrients, another key function of the intestine is to regulate the trafficking of environmental antigens across the host mucosal barrier.[1] Intestinal tight junctions (TJ) are responsible for the paracellular trafficking of macromolecules; therefore, they contribute to the balance between tolerance and immune response to non-self antigens.[1] Although considerable knowledge exists about TJ ultrastructure, relatively little is known about their pathophysiological regulation leading to local and/or systemic inflammation. Technologies that are capable to restore intestinal barrier function and, therefore, proper antigen trafficking may represent an innovative approach to prevent and/or treat immune-mediated diseases in which increased intestinal permeability seems to be an integral part of their pathogenesis.

What Are the Findings

Regulation of Intestinal Permeability: The Zonulin Pathway

In the past decade we have focused our research effort on the discovery of physiological modulators of intestinal TJ. Our studies led to the discovery and characterization of zonulin as the only human protein discovered to date that is known to reversibly regulate intestinal permeability by modulating intercellular TJ[2] (Figure 1). Through proteomic analysis of human sera, we have recently identified zonulin as pre-haptoglobin (HP) 2,[3] a molecule that, to date, has only been regarded as the inactive precursor for HP2, one of the two genetic variants (together with HP1) of human HPs. Our data suggest that pre-HP2 is a multifunctional protein that, in its intact single-chain form (ie, zonulin), regulates intestinal permeability caused by epidermal growth factor receptor transactivation through proteinase activated receptor 2,[3] whereas in its cleaved two-chain form, it acts as a hemoglobin scavenger.

Environmental Stimuli Causing Intestinal Zonulin Release

Among the several potential intestinal stimuli that can trigger zonulin release, small intestinal exposure to bacteria and gluten are the 2 triggers that have been identified so far.[2] Enteric infections have been implicated in the pathogenesis of several pathologic conditions, including allergic, autoimmune, and inflammatory diseases, by causing impairment of the intestinal barrier. We have generated evidence that small intestines exposed to enteric bacteria secreted zonulin.[2] This secretion was independent of the virulence of the microorganisms tested, occurred only on the luminal aspect of the bacteria-exposed small intestinal mucosa, and was followed by an increase in intestinal permeability coincident with the disengagement of the protein zonula occludens 1 from the tight junctional complex.[4] This zonulin-driven opening of the paracellular pathway may represent a defensive mechanism, which flushes out microorganisms so contributing to the innate immune response of the host against bacterial colonization of the small intestine.

Besides bacterial exposure, we have shown that gliadin, the main staple protein in wheat, also affects the intestinal barrier function by releasing zonulin by engaging the chemokine receptor CXCR3.[5] Our data demonstrate that in the intestinal epithelium, CXCR3 is expressed at the luminal level, is overexpressed in celiac disease (CD) patients, colocalizes with specific gliadin peptides, and that this interaction coincides with recruitment of the adapter protein, MyD88, to the receptor.[5]

Abbreviations used in this paper: **BBDP, BioBreeding diabetic prone; CD, celiac disease; HP, haptoglobin; TJ, tight junctions; T1D, type 1 diabetes; Zot, zonula occludens toxin.**

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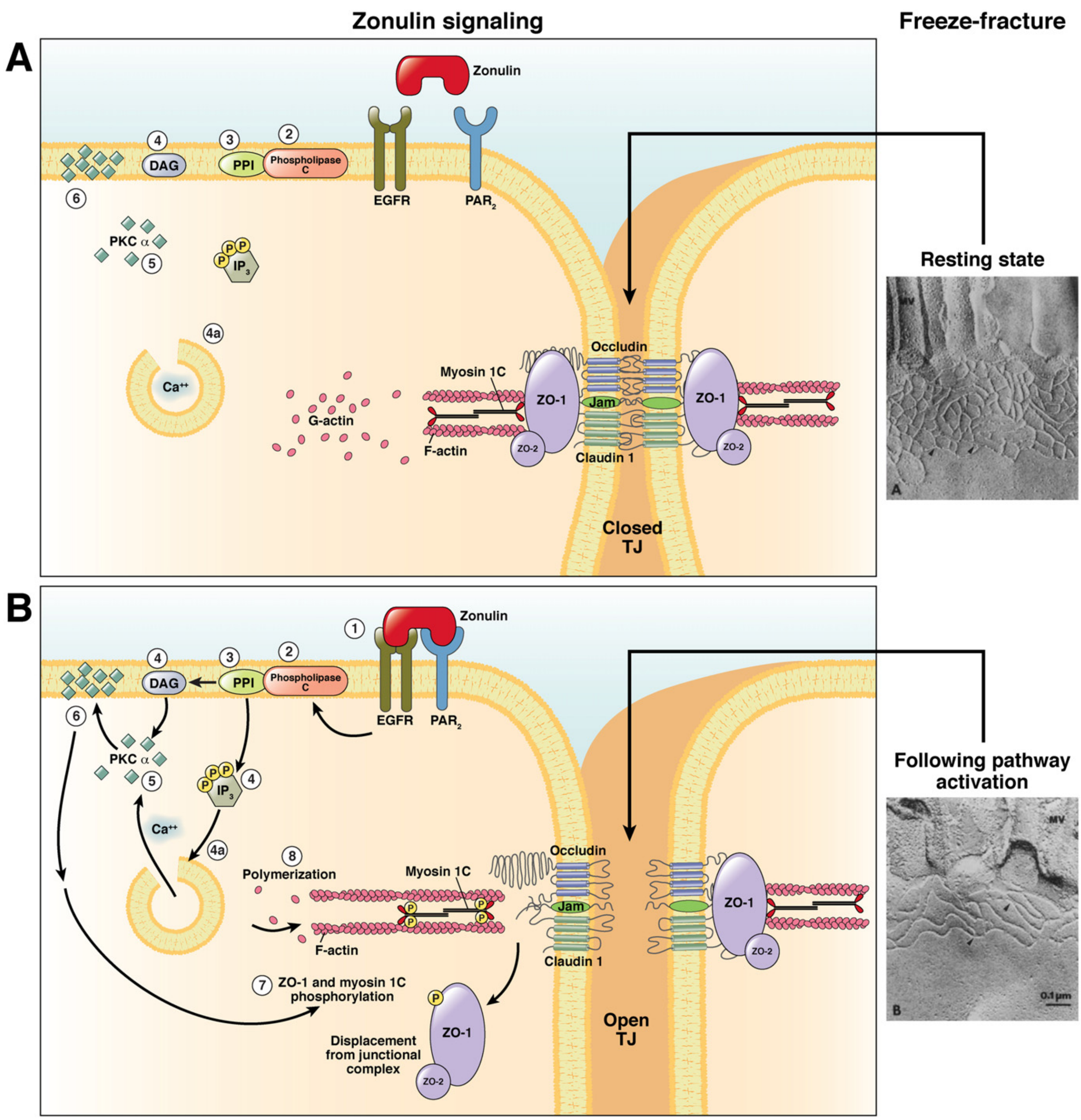

Figure 1. Schematic representation of zonulin mechanism of action. (*A*) Resting state: during the resting state, TJ proteins are engaged in both homophilic and heterophilic protein–protein interactions that keep TJ in a competent state closed as reflected by the complexity of TJ meshwork shown in the freeze-fracture electron microscopy photograph. (*B*) After zonulin pathway activation: zonulin transactivates epidermal growth factor receptor (EGFR) through proteinase activated receptor 2 (PAR_2) (*1*). The protein then activates phospholipase C (*2*) that hydrolyzes phosphatidyl inositol (PPI) (3) to release inositol 1,4,5-triphosphate (IP-3) and diacylglycerol (DAG) (*4*). Protein kinase C (PKC) α is then activated (*5*) either directly (via DAG) (*4*) or through the release of intracellular Ca^{2+} (via IP-3) (*4a*). Membrane-associated, activated PKC α (*6*) catalyzes the phosphorylation of target protein(s), including zonula occludens 1 (ZO-1) and myosin 1C, as well as polymerization of soluble G-actin in F-actin (*7*). The combination of TJ protein phosphorylation and actin polymerization causes the rearrangement of the filaments of actin and the subsequent displacement of proteins (including ZO-1) from the junctional complex (*8*). As a result, intestinal TJ become looser (see freeze-fracture electron microscopy). Once the zonulin signaling is over, TJ resume their baseline steady state.

Why Is This Important?

Shift of Paradigm in the Pathogenesis of Autoimmune Diseases

It is generally accepted that it is the interplay between environmental factors and specific susceptibility genes that underlies the aberrant immune response responsible for the onset of these diseases. Less than 10% of those with increased genetic susceptibility progress to clinical disease, suggesting a strong environmental trigger in the predisease state.[1] Environmental factors are also likely affecting the outcome of the process and the rate of progression to disease in those who develop pathologic outcomes. One theory is that antigens absorbed through the gut may be involved.[1]

A common denominator of autoimmune diseases is the presence of several preexisting conditions leading to an autoimmune process. The first is a genetic susceptibility for the host immune system to recognize, and potentially misinterpret, an environmental antigen presented within the gastrointestinal tract. Second, the host must be exposed to the antigen. Finally, the antigen must be presented to the gastrointestinal mucosal immune system after its paracellular passage (normally prevented by the TJ competency) from the intestinal lumen to the gut submucosa. In many cases, increased permeability appears to precede disease and causes an abnormality in antigen delivery that triggers the multiorgan process leading to the autoimmune response.

Therefore, the following novel hypothesis can be formulated to explain the pathogenesis of autoimmune diseases that encompasses the following 3 key points:

1. Autoimmune diseases involve a miscommunication between innate and adaptive immunity.
2. The classic autoimmune theories, molecular mimicry or bystander effect alone, may not explain entirely the complex events involved in the pathogenesis of autoimmune diseases. Rather, the continuous stimulation by non-self antigens (environmental triggers) appears necessary to perpetuate the process. This concept implies that the autoimmune response can be theoretically stopped and perhaps reversed if the interplay between autoimmune predisposing genes and trigger(s) is prevented or eliminated.
3. In addition to genetic predisposition and the exposure to the triggering non-self antigen, the third key element necessary to develop autoimmunity is the loss of the protective function of mucosal barriers that interface with the environment (mainly the gastrointestinal mucosa).

Role of Zonulin in Autoimmune Diseases

Celiac disease. CD represents the best testimonial of this theory. CD is a unique model of autoimmunity in which, in contrast to most other autoimmune diseases, a close genetic association with HLA genes, a highly specific humoral autoimmune response against tissue transglutaminase, and, most importantly, the triggering environmental factor (gliadin) are all known.[6] Early in the disease, TJ are opened as a result of zonulin up-regulation directly induced by the exposure to the disease's antigenic trigger gliadin, causing an increased paracellular passage of antigens, including gliadin, in the gut submucosa.[6] In genetically predisposed individuals this, in turn, triggers the gluten-specific adaptive immune response causing the autoimmune insult of the intestinal mucosa seen in patients with CD.[6] Once gluten is removed from the diet, serum zonulin levels decrease, the intestine resumes its baseline barrier function, the autoantibody titers are normalized, the autoimmune process shuts off, and, consequently, the intestinal damage (which represents the biological outcome of the autoimmune process) heals completely.

Type 1 diabetes. Recent studies have shown that altered intestinal permeability occurs in both type 1 and type 2 diabetes before the onset of complications.[7] This has led to the suggestion that an increased intestinal permeability due to alteration in intestinal TJ is responsible for the onset of diabetes. This hypothesis is supported by studies performed in an animal model that develops type 1 diabetes (T1D) spontaneously that showed an increased permeability of the small intestine (but not of the colon) of the BioBreeding diabetic prone (BBDP) rats that preceded the onset of diabetes by at least a month.[8] Furthermore, histologic evidence of pancreatic islet destruction was absent at the time of increased permeability but clearly present at a later time.[2,8] Therefore, these studies provided evidence that increased permeability occurred before either histologic or overt manifestation of diabetes in this animal model. We confirmed these data by reporting in the same rat model that zonulin-dependent increase in intestinal permeability precedes the onset of T1D by 2–3 weeks.[2] This goal was achieved by using the zonulin inhibitor AT1001 (subsequently used for clinical trial with the name larazotide acetate), an ectapeptide that prevents both zonulin-induced and zonula occludens toxin (Zot)–induced TJ disassembly (zonula occludens toxin is a zonulin prokaryotic counterpart elaborated by *Vibrio cholerae*).[9] Oral administration of AT1001 to BBDP rats blocked autoantibody formation and zonulin-induced increases in intestinal permeability, reducing the incidence of diabetes by 70%.[10] These studies suggest that the zonulin-dependent loss of intestinal barrier function is one of the initial steps in the pathogenesis of T1D in the BBDP animal model of the disease.[10] The involvement of zonulin in T1D pathogenesis was corroborated by our studies in humans showing that ~50% of T1D patients have elevated serum zonulin levels that correlated with increased intestinal permeability.[11] We also provided preliminary evidence suggesting that, as in the BBDP rat model of the disease, zonulin up-regulation precedes the onset of diabetes in T1D patients.[11] Recently, we have also reported a direct link between antibodies to Glo-3a (a wheat-related protein), zonulin up-regulation, and islet autoimmunity in children at increased risk for T1D.[12] Similarly to CD, these data link exposure to gluten and subsequent zonulin up-regulation to impairment of gut barrier function and subsequent onset of T1D in genetically predisposed individuals.

Zonulin, Gut Inflammation, Obesity, and Insulin Resistance

Recent evidence suggests a possible role of gut intestinal permeability in obesity.[7] In obese patients, intestinal permeability parameters are correlated to metabolic syndrome risk factors, obesity-induced inflammation, and nonalcoholic fatty liver disease.[7] More recently, it has been reported that zonulin is associated with obesity-associated insulin resistance.[13] Interestingly, circulating zonulin increased with body mass index, waist-to-hip ratio, fasting insulin, fasting triglycerides, uric acid,

Table 1. Major Diseases Associated With Zonulin (Pre-HP2) Biomarker

1. Autoimmune diseases
   - Ankylosing spondylitis
   - Celiac disease
   - Inflammatory bowel disease (Crohn's disease)
   - Rheumatoid arthritis
   - Systemic lupus erythematosus
   - Type 1 diabetes
2. Cancers
   - Brain cancers (gliomas)
   - Breast cancer
   - Lung adenocarcinoma
   - Ovarian cancer
   - Pancreatic cancer
3. Diseases of the nervous system
   - Chronic inflammatory demyelinating polyneuropathy
   - Multiple sclerosis
   - Schizophrenia

and interleukin-6. This last observation is of particular interest, because it suggests that the relationship between insulin sensitivity and circulating zonulin might be mediated through the increase of the obesity-related circulating interleukin-6, a cytokine that regulates zonulin expression by interacting with its gene promoters through signal transducer and activator of transcription 3 (STAT 3) activation.[13]

Role of Zonulin in Other Immune-Mediated Diseases

Since the discovery of zonulin as the precursor of HP2, this protein has been used as a biomarker of several immune-mediated diseases. Table 1 summarizes the 3 major categories of immune-mediated conditions, namely autoimmune diseases, tumoral diseases, and neuroinflammatory diseases, in which zonulin is up-regulated.

How Can This Be Translated in Routine Clinical Diagnosis/Treatment?

Zonulin as a Diagnostic Tool

With the appreciation that zonulin is associated to a series of immune-mediated diseases, a quantitative sandwich enzyme-linked immunosorbent assay has been developed to use its serum levels as a biomarker of intestinal barrier integrity in several inflammatory diseases.[3] Furthermore, it has been reported that carrying the HP2 allele (alias, zonulin gene) correlates with higher risk of developing inflammatory diseases, and HP2 homozygosis (2 copied of the zonulin gene) is associated with increased morbidity. We have recently developed a single-step reverse transcription–polymerase chain reaction protocol by using specific primers to amplify both HP1 and HP2 genes to perform zonulin genotyping to be correlated with the risk and severity of immune-mediated diseases.

Zonulin Inhibitor Larazotide for the Treatment of Autoimmune Diseases

CD and T1D autoimmune models suggest that when the finely tuned trafficking of macromolecules is deregulated because of a leaky gut, autoimmune disorders can occur in genetically susceptible individuals.[1,2] This theory implies that removing any of the 3 key elements function should block the autoimmune process. To challenge this hypothesis, zonulin inhibitor larazotide acetate was used with encouraging results in the BBDP rat model of autoimmunity.[2] Besides preventing the loss of intestinal barrier function, the appearance of autoantibodies, and the onset of disease, pretreatment with larazotide acetate protected against the insult of pancreatic islets and, therefore, of the insulitis responsible for the onset of T1D.[2] This proof-of-concept in an animal model of autoimmunity provided the rationale to design human clinical trials in which larazotide acetate was initially tested in an inpatient, double-blind, randomized placebo-controlled trial to determine its safety, tolerability, and preliminary efficacy.[14] No increase in adverse events was recorded among patients exposed to larazotide as compared with placebo. After acute gluten exposure, a 70% increase in intestinal permeability was detected in the placebo group, whereas no changes were seen in the larazotide acetate group.[14] Gastrointestinal symptoms were significantly more frequent among patients in the placebo group as compared with the larazotide acetate group.[14] Larazotide acetate has now been tested in approximately 500 subjects, with excellent safety profile and promising efficacy as concerns protection against symptoms caused by gluten exposure in CD patients.[15]

What Are the Roadblocks and/or Limitations?

The role of intestinal permeability in the pathogenesis of immune-mediated diseases is a relatively new field of translational science that only recently has received proper attention. Although the zonulin pathway is the only physiological mechanism described so far, it is likely that other pathways are involved in physiological TJ modulation. The prophylactic efficacy of zonulin inhibitors in preventing disease status has been proved in both animal models and humans. However, its efficacy in treating already established diseases or in slowing down progression of disease states remains to be established. Translating basic observations to clinical diagnostic and therapeutic applicability requires a strong academia-industry partnership to secure proper know-how, expertise, and economic resources. In a volatile global economy and with National Institutes of Health funding suffering possible cuts, the major roadblock to bring the zonulin technology to clinical applicability remains the availability of funds to support proper clinical trials.

Conclusions

The discovery of zonulin has increased understanding of the intricate mechanisms that regulate the intestinal epithelial paracellular pathway and the role of intestinal permeability in health and disease. Zonulin can be used as a biomarker of impaired gut barrier function for several autoimmune, neurodegenerative, and tumoral diseases and can be a potential therapeutic target for the treatment of these devastating conditions.

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Reprint requests

Address requests for reprints to: Alessio Fasano, MD, Mucosal Biology Research Center, University of Maryland School of Medicine, 20 Penn Street, HSF II Building, Room S345, Baltimore, Maryland 21201. e-mail: afasano@mbrc.umaryland.edu; fax: (410) 706-5508.

Conflicts of interest

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