Recombinant mouse prion protein alone or in combination with lipopolysaccharide alters expression of innate immunity genes in the colon of mice

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ABSTRACT. The objectives of this study were to test whether recombinant mouse (mo)PrP alone or in combination with LPS or under simulated endotoxemia would affect expression of genes related to host inflammatory and antimicrobial responses. To test our hypotheses colon tissues were collected from 16 male mice (FVB/N strain) and mounted in an Ussing chamber. Application of moPrP to the mucosal side of the colon affected genes related to TLR- and NLR- signaling and antimicrobial responses. When LPS was added on the mucosal side of the colon, genes related to TLR, Nlrp3 inflammasome, and iron transport proteins were over-expressed. Addition of LPS to the serosal side of the colon up-regulated genes related to TLR- and NLR-signaling, Nlrp3 inflammasome, and a chemokine. Treatment with both moPrP and LPS to the mucosal side of the colon upregulated genes associated with TLR, downstream signal transduction (DST), inflammatory response, attraction of dendritic cells to the site of inflammation, and the JNK-apoptosis pathway. Administration of moPrP to the mucosal side and LPS to the serosal side of the colon affected genes related to TLR- and NLR-signaling, DST, apoptosis, inflammatory response, cytokines, chemokines, and antimicrobial peptides. Overall this study suggests a potential role for moPrP as an endogenous ‘danger signal’ associated with activation of colon genes related to innate immunity and antibacterial responses.

KEYWORDS. Ussing chamber, mouse cellular prion protein, gene expression, antimicrobial response, lipopolysaccharide, colon, mice

ABBREVIATIONS. LPS, lipopolysaccharide; PrP°C, cellular form of the prion protein; moPrP°C, mouse cellular prion protein; TLR, toll like receptor; NLR, NOD like receptor; DST, downstream signal transduction

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Received December 10, 2014; Revised January 26, 2015; Accepted February 10, 2015.
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INTRODUCTION

The prion protein (PrPC) is widely expressed in a number of tissues; however, its physiological functions are far from clear. There is strong evidence that PrPC plays a protective role during oxidative stress,1 myelin repair of Schwann cells,2 maintenance of long-term memory,3 and stem cell renewal.4 However, given that PrPC is abundant in the brain, lymphatic tissues, kidney, and intestine it suggests that it might play other physiological roles as well. The fact that PrPC is expressed in cell-cell junctional domains of intestinal enterocytes is of particular interest.5 Recently Petit et al.6 reported that PrPC plays a fundamental role in the maintenance of intestinal epithelial barrier functions and suggested that the protein might connect barrier integrity and susceptibility to intestinal inflammation. PrPC also was reported to have strong cytoprotective effects,7 and over-expression of PrPC in Tga20 transgenic mice provided protection against dextran sodium sulfate (DSS)-induced colitis.8 Recently PrPC was shown to act as an antimicrobial peptide. In particular, Pasupuleti et al.9 demonstrated that both the intact PrPC and N-terminal peptides derived from this molecule display membrane-disrupting effects on both Gram-negative and Gram-positive bacteria similar to those observed with the human antimicrobial peptide cathelicidin LL-37.

A contentious question in prion disease is whether exogenous prion proteins serve as activators of host innate immune responses or whether the immune system mounts a protective response against prions. Further, it is not known whether prions serve as danger signals or damage associated molecular pattern molecules (DAMPs), or as exogenous ligands or pathogen associated molecular pattern molecules (PAMPs) to the host.

Toll-like receptors (TLRs) are key pattern-recognition receptors (PRRs) that recognize both PAMPs and DAMPs. Interestingly deficiencies in TLR-4, the receptor that binds to Gram-negative bacterial LPS, have been shown to expedite the development of prion disease.10 In addition, mice with mutations in interferon-regulatory factor 3 (IRF3), which is a myeloid differentiation primary response protein 88 (MyD88)-independent transcription factor that is activated downstream of TLRs, show accelerated prion pathogenesis following intraperitoneal infection.10,11 Also, an increase in PrPC expression has been demonstrated during gastric infection by Helicobacter pylori,12 bacterial kidney infection,13 and inflammation of the skin.14

In a previous study we demonstrated that LPS from Escherichia coli 0111:B4 interacts with recombinant Syrian hamster PrP (shPrP) and converts the α-helix-rich protein into a β-sheet rich isoform that forms amyloid-like fibrils resistant to proteinase K.15 Furthermore this conversion occurs under physiological conditions. Implications of interaction of prion protein with LPS are not known. Based on our preliminary findings we hypothesized that recombinant mouse (mo)PrP alone or in combination with LPS might affect the expression of genes related to host immunity and antibacterial responses. We evaluated this hypothesis by measuring the expression of 84 genes related to inflammation and antibacterial responses using colon tissue derived from FVB/N mice placed in an Ussing chamber system and exposed to differing concentrations of PrP and/or LPS both on mucosal and serosal sides of the tissue.

RESULTS

Gene expression profiling

PrPC is widely expressed in the epithelial cells of the intestinal tract but the functions of prion protein in the GI tract are not understood yet. It is not clear also whether exogenous prion proteins serve as activators of host innate immune responses or whether the immune system mounts a protective response against prion proteins. To address these questions we added moPrP into the mucosal side of an Ussing chamber system and analyzed gene expression related to inflammation and antibacterial responses. The results of gene expression profiles are shown in Table 1. The network was
### Table 1. Antibacterial gene expression in mice colon according to the treatment. Results of gene expression are shown as fold change (FC) relative to CTR group

| Gene | Description | LPS | M | PrP | LPS | M | PrP | M | CTR |
|------|-------------|-----|---|-----|-----|---|-----|---|-----|
| **Toll-like Receptor Signaling** | | | | | | | | | |
| Tlr1 | Toll-like receptor 1 | 1.20** | 1.32** | 1.32** | 1.44*** | 1.32** | | | |
| Tlr6 | Toll-like receptor 6 | 2.87 | 1.69 | 1.69 | 3.46 | 4.21 | | | |
| Tlr9 | Toll-like receptor 9 | 7.76 | 2.11 | 4.19 | 3.31 | 3.11 | | | |
| **NOD-like Receptor Signaling** | | | | | | | | | |
| Nod1 | Nucleotide-binding oligomerization domain containing 1 | -1.05 | 1.67 | 1.68 | 1.45 | 3.32*** | | | |
| Nod2 | Nucleotide-binding oligomerization domain containing 2 | -1.06 | 1.67 | 1.68 | 1.45 | 3.32*** | | | |
| Nod2 | Nucleotide-binding oligomerization domain containing 2 | -1.06 | 1.67 | 1.68 | 1.45 | 3.32*** | | | |
| **Mitogen-activated protein kinase kinase** | | | | | | | | | |
| Map3k7 | Mitogen-activated protein kinase kinase 7 | -1.05 | 1.32 | 1.66*** | 1.44*** | 1.66** | | | |
| **Interferon regulatory factor** | | | | | | | | | |
| Irf5 | Interferon regulatory factor 5 | 3.75** | 2.11 | 1.32 | 1.15 | 1.15 | | | |
| **Mitogen-activated protein kinase** | | | | | | | | | |
| Mapk14 | Mitogen-activated protein kinase 14 | 1.19 | 1.04 | 1.05 | 1.44*** | 1.32** | | | |
| Mapk8 | Mitogen-activated protein kinase 8 | 1.20 | 1.32 | 1.32 | 1.44*** | 1.66 | | | |
| **Apoptosis** | | | | | | | | | |
| Card6 | Caspase recruitment domain family, member 6 | 1.92 | 1.32 | 2.08 | 2.29* | 2.54 | | | |
| Card9 | Caspase recruitment domain family, member 9 | 1.18 | 1.05 | 1.20 | 1.15 | 1.15 | | | |
| Casp1 | Caspase 1 | 1.20 | 1.32 | 1.32 | 1.44*** | 1.32** | | | |
| Casp8 | Caspase 8 | 1.20 | 1.32 | 1.32 | 1.44*** | 1.66 | | | |
| **Mitogen-activated protein kinase** | | | | | | | | | |
| Mapk8 | Mitogen-activated protein kinase 8 | 1.20 | 1.32 | 1.32 | 1.44*** | 1.32** | | | |
| Myd88 | Myeloid differentiation primary response gene 88 | -1.33** | 1.32** | 1.32** | 1.44*** | 2.29* | | | |
| **Inflammatory response** | | | | | | | | | |
| Myd88 | Myeloid differentiation primary response gene 88 | -1.33** | 1.32** | 1.32** | 1.44*** | 2.29* | | | |
| Nlrp3 | NLR family, pyrin domain containing 3 | 6.03*** | 4.16 | 4.17 | 2.85 | 2.67 | | | |
| Rela | Rel A | 1.50 | 1.66 | 1.33 | 1.44 | 1.32** | | | |
| Tlr3 | Toll-like receptor 3 | -1.05 | 1.69 | 1.67 | 1.46 | 1.46** | | | |
| Tollip | Toll-interleukin 1 receptor (TIR) domain-containing adapter protein | -1.20 | 1.05 | 1.32 | 1.05 | 1.05 | | | |

(Continued on next page)
Table 1. Antibacterial gene expression in mice colon according to the treatment. Results of gene expression are shown as fold change (FC) relative to CTR group (Continued)

| Gene\(^{\text{a}}\) | Description\(^{\text{a}}\) | LPS M | LPS S | PrP M | PrP M + LPS M | PrP M + LPS S |
|------------------|---------------------------|-------|-------|--------|---------------|---------------|
| **Cytokines and Chemokines** | | | | | | |
| Ccl3             | Chemokine (C-C motif) ligand 3 | 3.81  | 3.31* | 1.02  | 1.44          | 1.02          |
| Il18             | Interleukin 18             | 1.51  | 1.31  | 1.66  | 2.28          | 1.65*         |
| **Antimicrobial peptides** | | | | | | |
| Camp             | Cathelicidin antimicrobial peptide | 1.15  | 1.29  | 1.33  | -1.10         | 2.12*         |
| Slpi             | Secretory leukocyte peptidase inhibitor | 3.83  | 5.26  | 10.55* | 7.30          | 42.24***      |

Within a row, fold-change with subscript were different relative to CTR group * \( P \leq 0.05 \), ** \( P \leq 0.01 \), *** \( P \leq 0.001 \).

\(^{\text{a}}\)Gene symbol and description. **CTR**: Pyrogen-free water was added to the mucosal side of the chamber at 700 \( \mu \)L; **LPS M**: Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was added to the mucosal side of the Ussing chamber at final concentration of 100 \( \mu \)g/mL; **LPS S**: LPS was added to the serosal side of the Ussing chamber at final concentration of 100 \( \mu \)g/mL; **PrP M**: moPrP (29-232) was added to the mucosal side of Ussing chamber at final concentration of 100 \( \mu \)g/mL; **PrP M + LPS M**: moPrP and LPS were applied to the mucosal compartment at final concentrations of 100 \( \mu \)g/mL; **PrP M + LPS S**: moPrP (100 \( \mu \)g/mL) was added to the mucosal side LPS (100 \( \mu \)g/mL) was added to the serosal side of the chambers.
visualized using Cytoscape version 3.0 (Fig. 1). Every node represents a gene and gene expression data, whereas their P-values are integrated as node attributes. Indeed when moPrP was applied to the mucosal side of the chamber (PrP M), 6 genes were significantly affected by the treatment. Four of them related to NLR signaling were up-regulated including Irf5, Map3k7, Akt1, and Sugt1 (P ≤ 0.05, p ≤ 0.001, p ≤ 0.01, p ≤ 0.01, respectively) compared to the control (CTR) group. Casp1, which is related to apoptosis, was down-regulated (p ≤ 0.05) and the antimicrobial peptide Slpi exhibited a 10-fold upregulated expression (p ≤ 0.05) (Table 1).

Further network analyses were performed to detect putative functional modules. The jActiveModules plugin from Cytoscape identified connected sections of the network in which the nodes have significant p values with a Z-score = 3.2 (Irf5, Map3k7, Akt1, Sugt1, Slpi and Casp1). This analysis indicates a group of nodes that may be co-regulated, suggesting a module whose activity is influenced by the treatment of PrP on the mucosal side of the colon (PrP M, Fig. 1a). To assess if the nodes with a significant p value were enriched biological processes we used the Cytoscape BiNGO plugin. The results of BiNGO in PrP M vs. CTR, revealed significant enrichment for processes relating to regulation of JNK cascade, regulation of stress-activated protein kinase signaling cascade, regulation of cellular response to stress and regulation of MAPK cascade with 2 genes (Akt1 and Map3K7).

Bacterial endotoxin, a cell wall component of all Gram-negative bacteria, is widely accepted as a potent pro-inflammatory agent,
and is ubiquitously present in the gastrointestinal (GI) tract. In this study we evaluated the colon responses to external LPS so that we could compare between LPS alone treatment and combination of LPS and moPrP. Within 40 min of the experiment, application of LPS to the mucosal side of the colon (in the Ussing chamber) up-regulated the expression of 4 genes (Table 1) related to TLR signaling (Tlr6, Irf5; p ≤ 0.05 and p ≤ 0.01, respectively) and inflammatory response (Nlrp3 and Slc11a1; p ≤ 0.001 and p ≤ 0.05, respectively). The jActiveModules plugin did not identify any putative activated module in this treatment. On the other hand, the Gene Ontology (GO) enrichment analysis with BiNGO in 4 upregulated genes in the LPS M group (LPS administered to the mucosal side) vs CTR, revealed significant enrichment for processes related to response to bacteria, activation of macrophages, immune response, activation of cells involved in the immune responses, activation of leucocytes and cytokine production with 3 genes (Tlr6, Nlrp3, and Slc11a1).

Besides administration of LPS to the mucosal side we also tested the effects of LPS administered on the serosal side of the colon (LPS S). The reason for this treatment was to simulate the effects of LPS when it is translocated into the systemic circulation (i.e., during endotoxemia). Administration of LPS to the serosal side of the colon mounted in the Ussing chamber affected the expression of 9 genes. As it is shown in Table 1, TLR signaling genes Tlr9, Irf5, and Ripk1 were up-regulated (p ≤ 0.05). Moreover Nlrp3 and Tollip, which are related to the host immune response, were upregulated (p ≤ 0.05 and p ≤ 0.001, respectively). In addition, it was observed that chemokine gene Ccl3 (p ≤ 0.05) and Sugt1 were up-regulated (p ≤ 0.001). On the contrary, the NLR signaling gene Nod2 expression and Card9, which is involved in apoptosis, were downregulated (p ≤ 0.05). The jActiveModules plugin identified one putative activated module (Z-score = 4.15) containing 11 significant genes (Fig. 1c). The GO enrichment analysis with BiNGO in PrP M + LPS M of the Ussing chamber was associated with modified expression of 12 genes, where 11 of them were upregulated and only (Card9) was down-regulated (Table 1). Toll-like receptor (TLR) signaling genes Tlr1, Map3k7, and Akt1 were up-regulated (p ≤ 0.001) and so were the downstream signal transduction genes Mapk14 and Mapk8 (p ≤ 0.001). Additionally, 4 genes related to apoptosis Card6, Ira1, Nfkbia, and Jun were overexpressed (p ≤ 0.05, p ≤ 0.001, p ≤ 0.05, and p ≤ 0.01, respectively) while Card9 remained downregulated (p ≤ 0.001). The inflammatory response genes Tollip and Rac1 were also upregulated (p ≤ 0.001). The jActiveModules plugin identified one putative activated module (Z-score = 4.15) containing 11 significant genes (Fig. 1c). The GO enrichment analysis with BiNGO in PrP M + LPS M vs CTR, revealed the most significant enrichment for processes related to signal transmission via a phosphorylation event, intracellular protein kinase cascade, intracellular signal transduction, positive regulation of cellular metabolic processes, and intracellular signaling pathway with 9 genes Map3k7, Akt1, Ira1, Mapk14, Jun, Tlr1, Rac1, Nfkbia, Mapk8. No annotation was found for Sugt1 gene.

Treatment with moPrP on the mucosal side and LPS on the serosal side (PrP M + LPS S) affected the highest number of genes, causing a dramatic response in gene expression. Twenty-one genes were up-regulated and only Casp1 remained down-regulated. TLR-signaling genes, (Tlr1, Tlr6, Irf5, Map3k7, Ripk1,
Traf6), NLR-signaling genes (Nod1, Sugt1, Xiap), downstream signal transduction (DST) genes (Mapk14), apoptosis genes (Casp8, Irak1, Tnfrsf1a, Jun), inflammatory response genes (Myd88, Rela, Tirap, Tollip), a cytokine gene (Il18), and antibacterial peptides (Camp and Slpi) were all upregulated (Table 1 for p values). The jActiveModules plugin identified one putatively activated module (Z-score = 3.15) containing 7 significant genes, Rac1, Myd88, Traf6, Irak1, Mapk14, Casp8, and Casp1 (Fig. 1d). The GO enrichment analysis with BiNGO in PrP M+ LPS S vs CTR, revealed the most significant enrichment for processes relating to regulation of immune system, stress-activated protein kinase signaling cascade, response to peptidoglycan, cellular response to lipopolysaccharide, MAPKKK cascade, intracellular signal transduction, and cell surface receptor linked signaling.

In terms of pathways, as it can be observed in Figure 2, the presence of moPrP on the mucosal side of the murine colon affected antimicrobial peptides as well as NLR and TLR pathways. Combining moPrP with LPS either on the mucosal or serosal side, caused dramatic changes in activation of pathways related to cytokine and chemokines, inflammation, apoptosis, DST as well as NLR- and TLR-pathways.

**Protein measurements**

Data on 2 colon protein concentrations are shown in Figure 3. Concentrations of the Camp protein (Fig. 3a), were significantly greater in PrP M treatment compared to PrP M + LPS S (1.45 ± 0.05 ng/μL vs 1.18 ± 0.05 ng/μL; p < 0.05). Concentration of colon Slpi also was affected by the treatment (p < 0.05). The Slpi protein showed greater concentrations in mice due to LPS M treatment.

**FIGURE 2.** Colon pathways affected by treatments. AMP (Antimicrobial peptide); Cyt+ (Chem Cytokines and chemokines); Inflamm (Inflammation); Apo (Apoptosis); DST (Downstream signal transduction); NLR (NOD-like receptor); TLR (Toll like receptor).

**FIGURE 3.** Protein concentrations in ng/mL of (a) Camp and (b) Slpi in mice colonic tissues. CTR: Pyrogen-free water was added to the mucosal side of the chamber at 700 μL; LPS M: Lipopolysaccharide (LPS) from E. coli 0111:B4 was added to the mucosal side of the Ussing chamber at a final concentration of 100 μg/mL; LPS S: LPS was added to the serosal side of the Ussing chamber at a final concentration of 100 μg/mL; PrP M: moPrP (29-232) was added to the mucosal side of Ussing chamber at a final concentration of 100 μg/mL; PrP M + LPS M: moPrP and LPS were applied to the mucosal compartment at a final concentrations of 100 μg/mL; PrP M + LPS S: moPrP (100 μg/mL) was added to the mucosal side LPS (100 μg/mL) was added to the serosal side of the chambers.
compared to CTR, LPS S, PrP M, and PrP M + LPS M treatments (33.73 ± 2.77 ng/μL vs 9.01 ± 2.29 ng/μL, 19.31 ± 3.24 ng/μL, 16.04 ± 3.24 pg/μL and 19.23 ± 3.24 ng/μL respectively) (p < 0.05). In addition, the concentration of Slpi in the PrP M + LPS S was greater compared to the CTR group 22.47 ± 3.24 ng/μL vs 9.01 ± 2.29 ng/μL (Fig. 3b).

**DISCUSSION**

Although PrPC is widely expressed in the epithelial cells of the intestinal tract the functions of prion protein in the GI tract are not known yet. Several studies have used microarray analyses to understand the functions of PrPC in the brain and mounting evidence indicates that PrP causes alterations in the expression of various genes related to proteolysis, protease inhibition, cell growth, apoptosis, signal transduction, cell adhesion, and immune responses.16-18 Our hypothesis was that recombinant moPrP alone or in combination with LPS might affect the expression of intestinal tissue genes (i.e., colon genes) related to innate immunity and antibacterial responses in FVB/N mice using an Ussing chamber model. The reason for combining moPrP with LPS was that recently we demonstrated that LPS was able to convert moPrP instantly and under normal physiological and environmental conditions into a β-rich isoform and resistant to proteinase K, which is biophysically similar to the scrapie form of the prion protein (PrPSc).15

Indeed our results clearly showed that the administration of moPrP to the mucosal side of the colon up-regulated 5 genes (Irf5, Map3k7, Act1, Sugt1, including the antimicrobial peptide Slpi) and downregulated the Casp1 when compared to the CTR group. In addition, further analysis of the PrP M gene network revealed Map3k7 and Act1 as putative activated nodes. Furthermore, results from BiNGO analysis showed significant enrichment for processes relating to regulation of the JNK cascade, regulation of the stress-activated protein kinase signaling cascade, regulation of the cellular response to stress, and regulation of the MAPKKK cascade with genes Akt1 and Map3K7. Map3k7 functions as an upstream signaling molecule for MAPKs pathways,19 Mapk8 (JNK1), Mapk14 (p38) and other inflammatory genes.20 The role of MAPKs pathway in the prion disease is not clear. The range of JNK1 and p38 actions in the cell are very complex. Nordstrom et al.21 examined the involvement of JNK and p38 in the formation of PrPSc indicating that JNK and p38 inhibit the formation of PrPSc. They suggested that a balance between intracellular signaling cascades might regulate the replication of prion. Here we report that exogenous prion protein alone might activate the MAPKs cascade activities through up-regulation of Akt1 and Map3K7 expression in the mouse colon.

Application of moPrP to the mucosal side of the colonic tissue upregulated one gene related to NLR signaling (i.e., Sugt1). This gene has been suggested to be a potential biomarker of neuron degeneration in Alzheimer’s disease because a decrease in the number of Sugt1 immunopositive neurons was found in the cerebral cortex of Alzheimer’s patients.22

One of the most interesting effects of moPrP M was the overexpression of Slpi gene. Slpi is an antibacterial protein that is secreted constitutively and in a regulated manner by intestinal epithelial cells.23 Antibacterial properties have been attributed to Slpi as it suppresses bacterial growth and is able to kill both Gram-negative (E. coli) and Gram-positive (Staphylococcus aureus) bacteria.24 Our findings are in agreement with previous research that has suggested a potential role of PrP in the regulation of Slpi gene expression.25 Concentration of colon Slpi protein in the PrP M treatment did not show significant changes with respect to other treatments. These results may be due to post-transcriptional and post-transduction modification processes. In our experiment Slpi showed the highest level of expression in presence of moPrP alone or in combination with LPS thus making the relationship between moPrP and Slpi an interesting subject of further investigation. moPrP on the mucosal side down-regulated expression of Casp1, which is known to cleave and activates pro-IL-1β and pro-IL-18.26 These results suggest that moPrP is
responsible for diminishing pro-inflammatory processes.

Our data also showed that administration of LPS to the mucosal side of the colon tissue, placed in the Ussing diffusion chamber, over-expressed 2 genes related to TLR signaling, Tlr6, and the well-known inflammasome gene Nlrp3. Our data are in agreement with previous research that shows that LPS induces upregulation of Tlr-6 in murine macrophages.27 Of note is the up-regulation of Slc11a1 gene from the inflammatory response pathway, also known as Nramp1, when LPS was administered on the mucosal side of the colon (LPS M). The product of this gene plays an important role in removal of Fe²⁺ and other divalent metals from the microenvironment that are essential for microbial growth. This suggests that colon cells are responding to the presence of LPS by lowering the availability of Fe²⁺ and other metals to reduce the growth of pathogenic bacteria.

Applying LPS to the serosal side of the colon in the Ussing chamber resulted in the over-expression of Tlr9, Irf5, Ripk1, Nlrp3, Sugt1, and Tollip. Meanwhile, Nod2 and Nlrc4 expression was downregulated (Table 1). Interestingly in our study Nod2 expression was down-regulated, whereas Tlr9 was up-regulated, suggesting activation via the MyD88-dependent pathway of Irf5. In support of this result An et al. (2002)28 showed that when macrophages are stimulated with LPS, Tlr9 gene expression is upregulated within 1 h and this expression reaches peak levels after about 3 h. Overall the application of LPS to the serosal side of the colon (LPS S) was associated with the upregulation of inflammasome, TLR- and NLR-signaling, inflammatory responses as well as one chemokine.

Administration of moPrP and LPS to the mucosal side of the colon (PrP M + LPS M), up-regulated genes related to signal transmission via a phosphorylation event, the intracellular protein kinase cascade, intracellular signal transduction, positive regulation of cellular metabolic processes, and intracellular signaling pathways with 9 genes Map3k7, Akt1, Tlr1, Rac1, Mapk14, Mapk8, Jun, Ira1, and Nfkbia. It has been noted that Map3k7 and Akt1, are involved in the activation of pro-inflammatory responses29-31 and that Tlr-1 is important for the production of chemokines related to the migration of dendritic cells (DCs) to the site of inflammation. Tlr-1 signaling in the intestinal epithelium leads to the initiation of DC migration and ultimately the generation of protective Th-17 and IgA immunity against Gram-negative bacterial infection.32 Rac1, an upstream positive modulator of Tnf-α-induced JNK activation, plays an important role in the biological regulation of apoptosis in normal small intestinal epithelial cells.33 Our data showed that this gene was also upregulated. Moreover, Mapk14, Mapk8, and Jun all of which are part of the JNK pathway and Ira1, and Nfkbia which are involved in apoptosis were up-regulated by moPrP M + LPS M. Previously we showed that LPS interacts with moPrP and converts it into a β-rich aggregated isoform that is resistant to proteinase K.15 We speculate that aggregates of moPrP and LPS were probably endocytosed by the colon epithelial cells and potentially induced overexpression of JNK-signaling pathway. JNK activation leads to phosphorylation of transcription factors controlling the apoptotic process,34 thereby resulting in cell death.35 When added alone to the mucosal side of the colon, moPrP did not have an effect on any of the genes related to apoptosis. However, when moPrP was combined with LPS, it affected genes related to apoptosis and regulated cell death. It has been shown that a relatively severe chronic stimulation with LPS exacerbates neuronal death and motor neuron axon degeneration during chronic neurodegeneration.36,37 Our results suggest that the combination of moPrP and LPS on the mucosal side of colonic tissue enhances the apoptotic processes, potentially in an effort to shed intestinal epithelial cells overloaded with LPS. This observation deserves further consideration with regards to the potential role of LPS as a co-factor in prion diseases and neuronal cell death.

The combination of moPrP M + LPS S (i.e., moPrP on the mucosal side and LPS on the serosal side) produced a much broader gene expression response for the colonic tissue. A total of 22 genes and 7 signaling pathways were affected by this treatment. TLR-signaling genes, (Tlr1, Tlr6, Irf5, Map3K7, Ripk1,
Traf6), NLR-signaling genes (Nod1, Sugt1, Xiap), DST genes (Mapk14), apoptosis genes (Casp8, lra1, Tnfrsf1a, Jun), inflammatory response genes (Myd88, Rela, Tirap, Tollip), a cytokine gene (IL18), and antibacterial peptides (Camp and Slpi) were all upregulated. NLR signaling plays a key role in recognizing PAMPs in the intracellular compartment and induces innate immune responses through cytosolic recognition of PAMPs. Once activated, Nod1 induces gene transcription through NF-κB and MAPK signaling pathways. Moreover the death-inducing signaling complex (DISC), which is mediated by the genes Tnfrsf1a and Casp8, and Jun were also up-regulated. This indicates that when moPrP was on the mucosal side and LPS on the serosal side, apoptotic signaling took place. Casp8 has been demonstrated to play a key role in mediating the Fas-induced extrinsic apoptotic pathway. Apoptosis is a vital mechanism in all multicellular organisms allowing them to readily shed cells in the GI tract. Therefore it appears that when moPrP was applied to the mucosal side of the colon tissue and LPS on the serosal side, apoptotic signaling took place. Casp8 expression remained downregulated and IL18 was upregulated showing that IL18 can also be intracellularly activated by a caspase-1-independent mechanism.

In addition, the expression of the antibacterial peptides Slpi and Camp was up-regulated by moPrP M + LPS S compared to the control group. The results of Slpi protein measurement in this treatment are in agreement with the gene expression data. This indicates that moPrP in combination with LPS might induce antibacterial and protective activities possibly through the up-regulation of Slpi and Camp. Beyond the previously mentioned functions of Slpi, Camp (cathelicidin antimicrobial peptide) is an important antibacterial compound produced by intestinal epithelial cells that has been shown to be important for survival of mice infected with E. coli O157:H7. In agreement with Hyeon (2012) it is postulated that moPrP might play a role in induction of apoptosis and in the activation of antibacterial responses in the presence of LPS or other DAMPs in the microenvironment.

Overall, the results from this study showed that applying moPrP to the mucosal side of the colon of male FVB/N mice using an Ussing chamber system induces expression of genes related to the JNK and p38 signaling cascade. In addition, moPrP strongly upregulated expression of the antimicrobial peptide Slpi, which suggests that the prion protein might serve as a "danger signal" for the presence of bacteria. The only gene down-regulated by moPrP M was Casp1, implying another role for moPrP in the negative control of inflammation. Application of moPrP and LPS to the mucosal side of the colon led to the over-expression of genes involved in the activation of pro-inflammatory responses and the attraction of DCs to the site of inflammation. Interestingly this treatment induced genes that are associated with the apoptosis of intestinal epithelial cells. Administration of moPrP to the mucosal side and LPS to the serosal compartment altered the highest number of genes (i.e., 22). These were related to TLR- and NLR-signaling, which are involved in the recognition of exogenous and endogenous bacterial molecules and the initiation of innate proinflammatory responses. Combinations of these treatments also induced genes related to apoptosis and antimicrobial peptides (Camp and Slpi).

More research is warranted to better understand the physiological functions of the prion protein in the GI tract. In particular, further examination of its role in the intestinal tract and in the activation of innate immunity and antibacterial responses in the GI tract are required. This work also suggests a possible role of LPS as a potential co-factor of moPrP in inducing intestinal epithelial cell death.

**MATERIALS AND METHODS**

Animals and experimental design. To test our hypotheses a total of 16 male mice of the FVB/N strain were used to collect colon samples for 2 consecutive experiments (n = 8 per experiment). The data from these 2 consecutive experiments are shown in Table 1. The reason...
for conducting 2 experiments was that the length of the mouse colon isolated from these animals was not sufficient to accommodate all the planned treatments. Mice were euthanized at age of 67 ± 7 days. All mice were fed the same diet before euthanization. The diet composition is shown in Table 2. Mice were all healthy with no clinical signs of disease prior to euthanasia. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Health Sciences and all the animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care.45

Following euthanasia, each colon (9-12 cm) was removed, and washed free of feces with Krebs buffer (Table 3). All the colons were immersed into a Krebs buffer solution and mounted into a thermostated (37°C) Ussing chamber system (6 ports in parallel per chamber), EasyMount Diffusion Chambers (Harvard Apparatus, Montreal, QC, Canada) within 40 min from the time of collection. The colon tissues (approximately 1.5 cm²) were mounted in different Ussing chambers to expose a tissue area of 0.3 cm². Krebs buffer solution (7 mL) with an optimal pH (i.e., 7.4) for murine colon tissue was added to the mucosal and serosal sides of the Ussing chamber. The chambers in these studies were gassed continuously with 95% O₂ and 5% CO₂, and the temperature kept at 37°C. Colonic tissues were allowed to equilibrate for 15 min prior to initiating the experiments. The electrophysiological responses and ionic fluxes were measured during the entire experimental procedure using a VCC MC6 multichannel voltage/current clamp and A&A software (data not shown; Harvard Apparatus, Montreal, QC, Canada). The entire Krebs solution was collected on both compartments of the Ussing chamber 40 min after the initiation of the experiment. All colonic tissues were collected at the end of the experimental procedures and stored at −86°C until gene expression analyses were performed.

Two consecutive experimental setups were established (n = 8 mice per experimental setup). In these experiments, the mucosal compartments of the chambers in the control groups (CTR) were supplemented with 700 μL of pyrogen-free dH₂O. Treatment 1 consisted of adding 700 μL of a pyrogen-free dH₂O solution containing 1 mg/mL of LPS from *Escherichia coli* O111:B4 strain (Sigma-Aldrich Co, Oakville, ON, Canada) on the mucosal side of the chamber. The chambers in these studies were gassed continuously with 95% O₂ and 5% CO₂, and the temperature kept at 37°C. Colonic tissues were allowed to equilibrate for 15 min prior to initiating the experiments. The electrophysiological responses and

| Table 2. Composition of diet fed to the mice |
|----------------|------------|
| Nutrient       | Amount (%) |
| Protein        | 20         |
| Fat (ether extract) | 5         |
| Fat (acid hydrolysis) | 5.6       |
| Total Saturated Fatty Acids | 0.93       |
| Total Monosaturated Fatty Acid | 0.99     |
| Fiber (Crude)   | 4.7        |
| Nitrogen-Free Extract (by difference) | 52.9      |
| Total Digestive Nutrients | 76.2      |
| Gross Energy, kcal/gm | 4.07     |
| Physiological fuel Value, kcal/gm | 3.41      |
| Metabolizable Energy, kcal/gm | 3.07      |
| Ash            | 6.1        |

| Table 3. Composition of Krebs buffer solution |
|----------------|------------|
| Composition    | Concentration (mM) | Amount (g) |
| NaCl           | 117         | 6.83748    |
| KCl            | 4.8         | 0.35784    |
| CaCl₂          | 2.5         | 0.367525   |
| MgCl₂          | 1.2         | 0.243972   |
| NaHCO₃         | 25          | 2.10025    |
| NaH₂PO₄        | 1.2         | 0.143952   |
| Glucose        | 11          | 1.98176    |
700 μL of a pyrogen-free dH2O solution containing moPrP at 1 mg/mL was added to the mucosal compartment (PrP M + LPS S). Before adding 700 μL of the treatment solution the same amount was removed from the same side of the Ussing chamber. Final concentrations of moPrP and LPS in the Ussing chambers for all treatments in both experiments were 100 μg/mL each. Colon samples from each treatment were collected after 40 min and analyzed.

**RNA extraction and real-time PCR**

Total RNA was extracted from all colonic tissues using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). The concentration and purity of the RNA was determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Mississauga, ON, Canada). To eliminate the possible amplification of contaminating genomic DNA, DNase treatment was carried out and total RNA (1 μg) was reverse-transcribed to cDNA using the RT^2 First Strand Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. The expression profiling of 84 key innate immune response genes in all colon tissues was determined by qPCR using a StepOnePlus ABI Prism platform (Applied Biosystems, Burlington, ON, Canada) and the Mice Antibacterial Response PCR Array Kit (Qiagen, Mississauga, ON, Canada). The kit measures 84 genes involved in signaling pathways such as toll-like receptor (TLR) signaling, nod-like receptor (NLR) signaling, bacterial pattern recognition receptors (PRRs), downstream signal transduction (DST), apoptosis, inflammatory response, cytokines and chemokines, and antimicrobial peptides. The kit contains primers for 84 genes and 12 controls (5 housekeeping genes including Actin β (Actb), Beta-2 microglobulin (B2m), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Glucuronidase β (Gusb), Heat shock protein 90 α (cytosolic), class B member 1 (Hsp90ab1) as well as genomic DNA contamination-, reverse transcription-, and positive PCR-controls. The qPCR was carried out in a total of 25 μL PCR mixture, containing RT^2 SYBR Green Rox™ qPCR Master Mix (Qiagen, Mississauga, ON, Canada) and cDNA, according to the manufacturer’s recommendations. The amplification conditions were an initial step of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of the amplification products was determined using a melting curve. qPCR data were normalized to the housekeeping genes and quantified using the delta-delta comparative threshold (Ct) method (ΔΔCt), employing an analysis tool from Qiagen. Normalized qPCR data were transformed from fold-change and presented in n-fold regulation relative to the CTR group according to the manufacturer’s recommendation.

Gene expression data were used to visualize the molecular changes that occurred for each treatment and network visualization was achieved by means of Cytoscape® (www.cytoscape.org) version 3.0.46 Analysis of network features was carried out using the jActiveModules plugin which identifies connected sections of the network in which the nodes have significant p-values. A module was considered significant when Z-score was greater than 3. This indicates a group of nodes that may be co-regulated, suggesting a module whose activity is influenced by the experimental context of the expression data. To determine which Gene Ontology (GO) terms were significantly over-represented in a set of genes Biological Networks Gene Ontology tool (BiNGO) was used.

**Protein analysis**

Commercially available ELISA kits were used to determine concentrations of proteins CAMP and SLPI in the colon samples (CAMP, BlueGene Biotech Co., Ltd., Shanghai, China, and SLPI Wuhan EIAab Science Co., Ltd. Wuhan, China). The sensitivity of the assay for CAMP measurement was 0.1 ng/mL. According to the manufacturer, the minimum detection limit of the assay for SLPI was 7.8-500 pg/mL as defined by the linear range of standard curves. All samples were tested in duplicate, as described by the manufacturer. Briefly,
standards and samples were incubated in a coated plate, followed by washing and addition of 100 μL during 2 h, followed by removing the liquid and addition of 100 μL of detection antibody for 1 h. The incubation with each of these reagents was followed by multiple washings with washing buffer (CAMP, 5 times full well, and SLPI 3 times adding 350 μL of washing buffer). The washing procedure was followed by adding 100 μL of secondary antibodies in the SLPI test. The optical densities were measured at 450 nm with a microplate spectrophotometer (Spectramax 340PC384 Molecular Devices Corporation, Sunnyvale, CA).

Statistical Analyses. Gene expression data are expressed as fold change compared to the CTR group. All statistical analyses were performed using Microsoft Excel software following manufacturer’s recommendations (www.sabioscience.com). Data for protein concentrations are expressed as mean value and SEM and were analyzed using the mixed procedures of SAS (SAS Institute Inc., Cary, NC) according to the model shown below:

\[ Y_{ij} = \mu + S_i + e_{ij} \]

Where \( Y_{ij} \) = observations for dependent variables, \( \mu \) = overall mean, \( S_i \) = fixed effect treatment, sequence \( i \) (i = 1 to 6), \( e_{ij} \) = random residual effect. Results were considered significant at \( P \leq 0.05 \).

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

**ACKNOWLEDGMENTS**

We are thankful to the staff of the Center for Prions and Protein Folding Diseases at the University of Alberta for providing the mice used for these experiments. The authors also wish to thank Ashenafi Abera for contributing to preparation and purification of the moPrP used in these experiments.

**FUNDING**

The authors would like to thank Alberta Livestock and Meat Agency Ltd. (ALMA), Alberta Prion Research Institute (APRI), and Natural Sciences and Engineering Research Council (NSERC) of Canada for providing financial support for this project.

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