Innate and adaptive immune effects of chicory root dietary fibers

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CHAPTER 3

TOLL-LIKE RECEPTOR 2 ACTIVATION BY β2→1 FRUCTANS PROTECTS BARRIER FUNCTION OF T84 HUMAN INTESTINAL EPITHELIAL CELLS IN A CHAIN LENGTH-DEPENDENT MANNER

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ABSTRACT

**Introduction:** Dietary fiber intake is associated with lower incidence and mortality from disease, but the underlying mechanisms of these protective effects are unclear. We hypothesized that β2→1-fructan dietary fibers confer protection on intestinal epithelial cell barrier function via Toll-like receptor 2 (TLR2), and we studied whether β2→1-fructan chain-length differences affect this process.

**Methods:** T84 human intestinal epithelial cell monolayers were incubated with 4 β2→1-fructan formulations of different chain-length compositions and were stimulated with the proinflammatory phorbol 12-myristate 13-acetate (PMA). Transepithelial electrical resistance (TEER) was analyzed by electric cell substrate impedance sensing (ECIS) as a measure for tight junction–mediated barrier function. To confirm TLR2 involvement in barrier modulation by β2→1-fructans, ECIS experiments were repeated using TLR2 blocking antibody.

**Results:** After preincubation of T84 cells with short-chain β2→1-fructans, the decrease in TEER as induced by PMA (62.3 ± 5.2%, P < 0.001) was strongly attenuated (15.2 ± 8.8%, P < 0.01). However, when PMA was applied first, no effect on recovery was observed during addition of the fructans. By blocking TLR2 on the T84 cells, the protective effect of short-chain β2→1-fructans was substantially inhibited. Stimulation of human embryonic kidney human TLR2 reporter cells with β2→1-fructans induced activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), confirming that β2→1-fructans are specific ligands for TLR2.

**Conclusions:** To conclude, β2→1-fructans exert time-dependent and chain length–dependent protective effects on the T84 intestinal epithelial cell barrier mediated via TLR2. These results suggest that TLR2 located on intestinal epithelial cells could be a target of β2→1-fructan–mediated health effects.
INTRODUCTION

3.1 Background

It is becoming more accepted that dietary fiber intake leads to a reduced incidence of disease (1-4) and related mortality (5-8). The mechanisms behind this effect of dietary fibers are still incompletely understood. Factors that were suggested to play an important role are improvement of the gut microbiota composition and the related SCFA profiles in the intestine (9-11). However, direct immune effects by ligand interaction of the fibers with so-called pattern recognition receptors on gut immune cells (12-15) were also suggested.

Another mechanism by which dietary fibers may contribute to health is by modulating the integrity of the intestinal epithelial barrier. A disrupted barrier is considered to play a role in the etiology and pathogenesis of several diseases (16), such as atopic eczema (17, 18), asthma (19, 20), inflammatory bowel disease (21, 22), diabetes (23-25), obesity (26, 27), celiac disease (28-30), and diarrhea-predominant irritable bowel syndrome (31, 32). A compromised intestinal barrier can be associated with hyperpermeability, also described as leaky gut syndrome (33). Disruption leads to increased translocation of bacteria, endotoxins, and other macromolecules, which can be important triggers for aberrant local or peripheral immune reactions (27, 34-36). A category of widely used dietary fibers is formed by β2→1-fructans. Depending on the polymer chain lengths, these fibers are also described as inulin, inulin-type fructans (ITFs), fructooligosaccharides, or oligofructose (10). β2→1-fructans are well studied for their beneficial effects on the gut microbiota and health (10, 37-41). Studies into the effects of β2→1-fructans on immune cells suggest that they exert direct effects by receptor–ligand interactions and subsequent cytokine production (15, 42). However, direct effects of β2→1-fructans on the barrier integrity of human intestinal epithelial cells are, to the best of our knowledge, not available. Previous studies from our group suggest that chain-length differences of β2→1-fructans should be taken into consideration when studying their signaling effects: shorter-chain β2→1-fructans induce a more anti-inflammatory cytokine profile in isolated human peripheral blood mononuclear cells compared with long-chain β2→1-fructans (15). Differences in chain length of β2→1-fructans may also induce different effects on epithelial cells. We investigated whether β2→1-fructans exert protective effects on barrier function of human intestinal epithelial cells. This was done by analyzing the effect of β2→1-fructans on transepithelial electrical resistance (TEER).
of T84 intestinal epithelial cell monolayers, damaged with the barrier-disruptive agent phorbol 12-myristate 13-acetate (PMA). The dynamics of this process were studied with regard to timing of fiber incubation and fructan chain-length effects. Because β2→1-fructans were identified recently as Toll-like receptor (TLR) ligands (15) and TLR2 is highly important in intestinal barrier regulation (43), the other aim of this study was to investigate whether receptor interactions with TLR2 on the epithelial surface are involved in β2→1-fructan-mediated barrier modulation.

METHODS

3.2 Experimental design

T84 intestinal epithelial cells were grown to differentiated monolayer stage, and resistance across the monolayer was continuously measured at multiple frequencies after different challenges. Measurements performed at 500 Hz specifically represent the tight junction (TJ) mediated resistance and these were used to calculate the AUC. To establish whether β2→1-fructans exert protective or recovery effects, a damage model was introduced based on challenge of the T84 cells with a known barrier disrupting agent, PMA (44). The rationale for the first experiment was to study whether β2→1-fructans protect T84 cells against PMA-induced loss of TEER, and whether β2→1-fructan chain length profile is important in TEER modulation. T84 cells were incubated for 24h, with four different formulations of β2→1-fructans of different average DP and DP profile (ITF I–IV) provided by Sensus B.V., Roosendaal, the Netherlands, followed by addition of PMA (10 nM, Sigma-Aldrich Chemie B.V., the Netherlands). The second experiment was designed to investigate the possible time dependency of the effect of β2→1-fructan incubation. Cells were stimulated in different order with different compounds; protocol I) incubation with PMA for 6h followed by removal of the medium and addition of β2→1-fructans in culture medium at a final concentration of 100 μg/mL, or protocol II) incubation for 24h with β2→1-fructans, followed by addition of PMA. This stimulation medium was left on the cells for at least 6h to allow the PMA to take effect. As the interval for recovery in protocol I, a fixed period of time was taken (12h) following removal of PMA medium. This period was based on the average recovery time of the cells treated with PMA followed by control medium, represented by TEER values returning to the initial value (100%). As a
measure for induced recovery, AUC was plotted for this time frame relative to the AUC of untreated control (without PMA), which was set to 100%. For protocol II, the interval for protection against the reduction of TEER was based on the time frame starting at the addition of PMA until the maximal decrease in TEER was reached (after 6h), and this fixed time frame was taken to calculate the AUC relative to untreated controls. With the third experiment, we aimed to study the role of TLR2 in β2→1-fructan mediated effects on T84 cells. To this end, T84 cells were incubated with culture medium or TLR2 blocking antibody (catalog # pab-hstlr2, 5 μg/mL, InvivoGen, Toulouse, France) for 10 min prior to addition of β2→1-fructans. Cells remained in this medium for 24h, followed by addition of PMA. The 6h time frame following PMA addition was used for AUC calculations. Finally, to confirm that β2→1-fructans signal through TLR2, HEK hTLR2 reporter cells were stimulated with ITFL-IV.

3.3 Investigational compounds
To study the effects of β2→1-fructans on intestinal epithelial cell barrier function, four different formulations were applied, based on the chemical characterization of their chain length profiles by High-Performance Anion-Exchange Chromatography and High Pressure Size Exclusion Chromatography (15). Endotoxin concentrations in the formulations were analyzed by Toxikon (Leuven, Belgium), and all fell below 0.3 x 10^{-3} endotoxin units (EU) μg^{-1}. Briefly, the average chain length of these formulations is as ITF I (Frutalose® OFP) < ITF II (Frutafit® CLR) < ITF III (Frutafit® HD) < ITF IV (Frutafit® TEX). ITF I is a fructooligosaccharide (FOS) compound, with chain lengths of ≤ DP10. ITF II is an inulin, enriched with FOS, with most chains shorter than DP10, but also containing chains with DPs up to 60. ITF III is ‘native’ inulin, and ITF IV can be described as a long chain enriched inulin. Both ITF III and IV consist predominantly of chains ranging from DP10 to DP60. Chain length profiles of the applied formulations are summarized in Figure 1.

3.4 T84 cell culture
T84 human colon carcinoma cells (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) were grown to ca. 80% confluency at 37°C, 5% CO₂ in culture medium consisting of 1:1 Ham’s F12 medium:DMEM, acquired premade from Sigma-Aldrich Chemie B.V.), supplemented with 10% HyClone FBS, Thermo Scientific, Breda, the Netherlands) and gentamicin (50 μg/mL, Life Technologies Europe B.V., Bleiswijk, the Netherlands). Cells were maintained as previously described (45). Trypsin was acquired from
3.5 Trans Epithelial Electrical Resistance measurements

Multiple electrode gold-plated 8 well chamber slides (8W10E, Applied Biophysics, IBIDI, München, Germany) were coated with 400 μL/well of a 0.2% L-cysteine (Sigma-Aldrich Chemie B.V.) solution in PBS for 30 min at room temperature. Wells were washed twice with PBS, and coated overnight at room temperature with 400 μL/well of 1% PureCol™ bovine tail collagen (Nutacon B.V., Leimuiden, the Netherlands) and 0.1% BSA (Sigma-Aldrich Chemie B.V.) in PBS. Wells were then washed twice with culture medium and cells were seeded at a density of 2×10⁴ cells per well in a final culture volume of 400 μL/well. Prior to stimulation, the cells were maintained in the wells for 14 days to reach a stable TEER. Medium was changed twice a week. The chamber slides were put into an ECIS incubator (Z-Theta model, Applied Biophysics, Troy, New York, USA) and resistance was measured continuously at multiple frequencies (46).
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3.6 HEK hTLR2 reporter cell culture and reporter assay
HEK hTLR2 reporter cells were cultured and NF-κB activity was determined as previously described (15). As positive control, the additional TLR2 agonist heat killed *Listeria monocytogenes* was applied by adding 20 µl solution to 180 µl of cell suspension (1x10⁸ bacteria/mL, InvivoGen).

3.7 Statistical analysis
Statistical analysis was performed using GraphPad Prism 5.0 software. D’Agostino & Pearson omnibus normality test was used to test for normal data distribution. Statistical significance levels were determined by one-way ANOVA and Tukey’s Multiple Comparison test or by Dunnett’s Multiple comparison test to compare treatment with controls. Results are expressed as mean ± SD. P-values < 0.05 were considered statistically significant.

RESULTS

3.8 β2→1-fructans exert chain length dependent protection against PMA-induced loss of barrier function in T84 intestinal epithelial cells
To study whether β2→1-fructans protect T84 cells against PMA-induced loss of TEER, and whether β2→1-fructan chain length profile is important in TEER modulation, T84 cells were incubated with four different formulations of β2→1-fructans of different average DP and DP profile, (ITF I–IV). AUC for the 6h time period following PMA addition was plotted for the different ITF treatments, as a percentage of the AUC of untreated control, which was set to 100 % (*Figure 2*). The damage model of PMA treatment induced a decrease in TEER, resulting in an AUC of 61.5 ± 5.8% (P<0.001) as compared to control. Strikingly, 24h of preincubation of T84 cells with 100 µg/mL of the ITF I or ITF II β2→1-fructans conferred a protective effect against PMA-induced loss of resistance (P<0.001 and P<0.01 respectively). ITF I conferred the strongest protection, with an AUC of 91.0 ± 6.6% of the control AUC, followed by ITF II, which rendered an AUC of 75.4 ± 3.2% of the control AUC. TEER values for treatment with the longer chain compounds (ITF III and IV) were not statistically different from TEER values as induced by PMA treatment, demonstrating that these compounds did not exert a protective effect. These results indicate that the protective effect of β2→1-fructans is a chain length dependent phenomenon, which is only conferred by the short chain formulations ITF I and ITF II.
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Figure 2. Chain length–dependent protection of transepithelial electrical resistance across T84 epithelial cell monolayers treated with different ITF formulations and PMA. AUC was plotted for the time range starting at the addition of PMA and after a 6-h time period. Values are means ± SDs, n = 3. Data are representative of 3 individual experiments. Statistical significance levels were determined with 1-factor ANOVA and Tukey’s multiple comparison test. Labeled means without a common letter differ, P < 0.05. ITF, inulin-type fructan; PMA, phorbol 12-myristate 13-acetate.

3.9 The protective effect of short chain β2→1-fructans against PMA-induced T84 barrier loss is time dependent

To establish if timing of short chain β2→1-fructan incubation is important in their functionality, two protocols for incubation were applied; I) preincubation of T84 cells with PMA for 6h followed by removal of the medium and addition of short chain β2→1-fructans (ITF I) in culture medium, and II) preincubation of T84 cells for 24h. with ITF I followed by addition of PMA (Figure 3). Figure 3A shows an example representative of relative TEER values obtained with protocol I, and the 12h interval used to calculate the AUC, which was subsequently plotted in figure 3B. Figures 3A and B show that protocol I did not induce TEER recovery effects as compared to PMA treatment. A representative example of relative TEER values obtained with protocol II, and the 6h interval used to calculate the AUC are plotted in figure 3C. Here, a significant protection was established, minimizing the decrease in TEER at 6h after PMA addition to 15.2 ± 8.8% (P<0.01), whereas PMA treatment alone induced a reduction of 62.3 ± 5.2% (P<0.001) of the initial TEER values. This protective effect was also observed when calculating the AUC (figure 3D). Over the 6h
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Figure 3. Time-dependent impact of different incubation protocols on TEER across T84 epithelial cell monolayers. Example of TEER as induced by protocol I (A). AUC for 12 h from the time point of PMA addition, representing the period of recovery for PMA-treated cells to 100% of the initial value for protocol I (B). Values are means ± SDs, n = 6. Example of TEER as induced by protocol II (C). AUC for 6 subsequent hours after addition of PMA plotted as percentage of the AUC of untreated controls (D). Values are means ± SDs, n = 6. Data are representative of 3 individual experiments, and data for ITF I are shown for n = 6. Statistical significance levels were determined with 1-factor ANOVA and Tukey’s multiple comparison test. Labeled means without a common letter differ, P < 0.05. ITF, inulin-type fructan; PMA, phorbol 12-myristate 13-acetate; TEER, transepithelial electrical resistance.

period, the AUC of PMA treatment was 69.5 ± 2.3% (P<0.001) of the AUC of untreated controls, whereas the AUC of cells pretreated with ITF I followed by PMA was 91.0 ± 6.6% (P<0.01) of the AUC of untreated controls. These results indicate that timing of incubation with β2→1-fructans is an important factor for protection of the T84 barrier function.

3.10 Blocking of TLR2 inhibits short chain β2→1-fructan mediated protection of TEER

Because β2→1-fructans have recently been identified as TLR ligands (15), and TLR2 is highly important in intestinal barrier regulation (43), we hypothesized that the protective effect of short chain β2→1-fructans on epithelial cells against PMA might be mediated through TLR2. T84 cells
were preincubated with normal culture medium or with TLR2 blocking antibody prior to 24h incubation with short chain β2→1-fructans, followed by PMA challenge (Figure 4). As the effects of longer chain β2→1-fructans did not induce protective effects, ITF III and IV were not included in the TLR2 blocking experiments. Figure 4A and B show representative examples of TEER values as induced by PMA, preincubation with IFT I and ITF II respectively, and pretreatment with the blocking antibody. AUC for a 6h time frame following PMA addition was plotted in figure 4C, showing that pretreatment with TLR2 blocking antibody significantly reduced the short chain β2→1-fructan mediated protection for both short chain formulations, with a TEER curve approaching the PMA curve. These results indicate that in T84 cells, TLR2 is involved in the protective mechanism of short chain β2→1-fructans against PMA.

![Figure 4](image-url)

**Figure 4.** Effects of blocking TLR2 on inulin-type fructan–mediated protection of T84 TEER. TEER of T84 cells with or without TLR2 blocking antibody (AB), incubation with ITF I for 24 h, and 10 nmol/L PMA (A). TEER of T84 cells with or without TLR2 blocking antibody (AB), incubated with ITF II for 24 h, and 10 nmol/L PMA (B). The AUC for 6 subsequent hours of each treatment (C). Data are representative of 3 individual experiments, and data for ITF I are shown for n = 9. Labeled means without a common letter differ, P < 0.05. Statistical significance levels were determined with 1-factor ANOVA and Tukey’s multiple comparison test. AB, antibody; ITF, inulin-type fructan; PMA, phorbol 12-myristate 13-acetate; TEER, transepithelial electrical resistance; TLR2, Toll-like receptor 2.
3.11 β2→1-fructans exert TLR2-mediated NF-κB activation in HEK hTLR2 reporter cells

TLR2 was suggested as a mediator of β2→1-fructan signaling in immune cells (15), and in previous studies an important role has been attributed to TLR2 in modulating barrier function (43, 47-49). To confirm the role of TLR2 in β2→1-fructan signaling, HEK hTLR2 reporter cells were incubated with a concentration series of β2→1-fructans of different chain lengths. β2→1-fructans induced TLR2-mediated NF-κB activation in the reporter cell line (Figure 5). Short chain β2→1-fructans (ITF I) induced a 2.8 ± 1.2 fold induction (P<0.05) of NF-κB/AP-1 activation compared with control, and with increasing mean fructan chain length, the fructans conferred stronger activation, up to 5.9 ± 0.5 fold induction (P<0.05) for the longest mean fructan chain formulation (ITF IV) as compared to control. These results confirm the role of TLR2 in β2→1-fructan signaling and indicate that fructan chain length is an important factor in determining the strength of the TLR2 response. Short chain β2→1-fructans conferred a moderate TLR2 activation, whereas the longer chain β2→1-fructans induced a relatively strong TLR2 response.
Discussion

To our best knowledge, this is the first time β2→1-fructans have been identified in their capacity as modulators of human intestinal epithelial cell barrier function through TLR2. Proof of principle was established that β2→1-fructan dietary fibers can protect the integrity of intestinal epithelial cell monolayers. This effect was observed for the shorter chain ITFs (ITF I and II) but not for the longer chain formulations (ITF III and IV). This is in accordance with a previous study in our group, in which chain length differences of the applied β2→1-fructans were important factors in inducing different effects on human immune cells with regard to cytokine profiles (15). In this study, the short chain β2→1-fructans induced a more anti-inflammatory cytokine pattern compared to the longer chain formulations, indicating chain length dependent differences in downstream effects in immune cells. In addition, two studies by Ito et al. performed in rats corroborated chain length dependent effects on immune cells as well as intestinal barrier function in vivo. The studies by Ito et al. demonstrated that β2→1-fructans stimulated intestinal immune parameters in a chain length dependent manner (50), and that short chain β2→1-fructans reduced translocation of endotoxins and bacteria in a TNBS-induced colitis model (51).

By using different timing protocols regarding short chain β2→1-fructan or PMA treatment we observed a protective effect of the fructans on TEER, provided that the cells were incubated with the fructans for 24h, before stimulation with PMA. Treatment with PMA followed by incubation with β2→1-fructans did not induce recovery effects, indicating that in this model, fructans exerted protective effects rather than effects on repair processes. This conclusion was based on the described model using low dose treatment. The rationale behind treatment with low dose fructans (100 µg/ml) was based on our previous results in peripheral blood mononuclear cells (15), where this dose already induced substantial cell activation in the form of production of several cytokines upon 24h of stimulation. In addition, several studies have shown that preincubating cells of different tissue types with TLR2 agonists can protect against detrimental effects of barrier disruptive agents or ischemia-reperfusion injury (47-49, 52, 53). In these studies, protection is often established under low dose stimulatory conditions.

With the current study, novel proof of principle was demonstrated for time dependent protective effects of β2→1-fructans on barrier function of human intestinal epithelial cells. Whether the critical time frame of
preincubation with β2→1-fructans can be reduced as compared to the 24h preincubation protocol while retaining a protective effect remains to be studied. This time frame may give an indication which sort of cellular processes are affected, such as receptor/adapter molecule assembly at the cell membrane, modulation of kinase activity, or further downstream effects such as nuclear translocation of messenger molecules, gene transcription, and protein expression. These results prompt further studies into the exact mechanisms behind the observed protective effects of the fructans on the epithelium.

Considering epithelial cells of the intestine specifically, TLR2 is an important player in regulating permeability and thus barrier function (43). The role of TLR2 in the protective action of β2→1-fructans on intestinal epithelial cells was confirmed with blocking experiments. In addition, the specific dynamics of β2→1-fructan mediated TLR2 activation were demonstrated. Besides possible fructan chain length-, or dose dependent effects on the strength of TLR2 activation in intestinal epithelial cells, differences in TEER modulation may be due to the ability of TLR2 to heterodimerize with TLR1 (54), TLR6 (54) or TLR10 (55), depending on the stimulus (43, 54), and the ability to signal together with a spectrum of different coreceptors (56). These features of TLR2 diversify the downstream effects of TLR2, and may provide an explanation for chain length induced differences between the different fructan formulations.

The discrepancy between the longer chains inducing strong TLR2 activation but not exerting a protective effect on barrier function, could be due to mechanistic differences in receptor interactions at the cellular surface (15). This type of mechanism has been previously described for TLR4 by Visintin et al. (57). They suggest that differences in agonist clustering mechanisms for TLRs culminate in enhanced signal transduction and different downstream reactions. This is in accordance with our previous observations that inulin-type fructans have different TLR activation patterns (15). We suggest that by activating different numbers of receptors at a time, and at different distance from each other on the cell membrane, long chain β2→1-fructans may induce a different cellular response than the short chain β2→1-fructans. However an in depth discussion of these pathways is beyond the scope of the present study, and the role of TLR2 dynamics in barrier function is subject of further research in our lab.

As an analogue of the endogenous second messenger diacylglycerol, PMA induces activation and translocation of protein kinase C (PKC), leading to elevated intracellular Ca$^{2+}$ and modulation of TEER (44). PMA
challenge of T84 cells can be viewed as a simplified model for dietary stimuli (58) or intestinal pathogens (59) involved in modulating PKC signaling and affecting the gut barrier. The read out for TLR2 activation in the HEK reporter cell line was analyzed by the production of SEAP upon NF-κB activation. In intestinal epithelial cells, TLR2 activation was shown to acts as an NF-κB inhibitor (43). As PMA can also target NF-κB, downstream of PKC-β1, via stabilization of IκBα (60, 61), inhibition of NF-κB could be one of the downstream mechanisms by which the epithelial barrier is partially protected by the fructans.

Although PKC was not studied in our experiments, results from our model suggest that short chain β2→1-fructans may interfere in this pathway, by partly inhibiting the cellular response to PMA. Previous studies with inulin have shown that their signaling can induce PKC activation in RAW 264.7 cells (60) and in rat distal colonic mucosa (62). However, the typical PKC dynamics are greatly dependent on the type of tissue and the organism which is studied (44). Moreover, the PKC family consists of different subclasses and several isoforms, each having distinct dynamics for activation and downstream effects (63-67). A study in T84 cells by Song et al. (68), showed that PMA induced PKCα translocation to the apical surface, which was correlated with a decreased TEER. Interestingly, PKCα activation has previously been linked to TLR2 signaling cascades in mouse and human dendritic cells (69).

In conclusion, β2→1-fructans may protect the integrity of the intestinal barrier from damage, by directly binding to TLR2. Since they also have immunomodulating effects and prebiotic effects, they form a promising category of dietary fibers with regard to intestinal health effects, and further studies into their effects upon ingestion on physiological, cellular, and molecular level are warranted.
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