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

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. 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. 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. 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

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)\(^9\), fructooligosaccharides, or oligofructose \(^{(10)}\). \(\beta_2\rightarrow 1\)-fructans are well studied for their beneficial effects on the gut microbiota and health \(^{(10,37–41)}\). Studies into the effects of \(\beta_2\rightarrow 1\)-fructans on immune cells suggest that they exert direct effects by receptor–ligand interactions and subsequent cytokine production \(^{(15,42)}\). However, direct effects of \(\beta_2\rightarrow 1\)-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 \(\beta_2\rightarrow 1\)-fructans should be taken into consideration when studying their signaling effects: shorter-chain \(\beta_2\rightarrow 1\)-fructans induce a more anti-inflammatory cytokine profile in isolated human peripheral blood mononuclear cells compared with long-chain \(\beta_2\rightarrow 1\)-fructans \(^{(15)}\). Differences in chain length of \(\beta_2\rightarrow 1\)-fructans may also induce different effects on epithelial cells.

We investigated whether \(\beta_2\rightarrow 1\)-fructans exert protective effects on barrier function of human intestinal epithelial cells. This was done by analyzing the effect of \(\beta_2\rightarrow 1\)-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 \(\beta_2\rightarrow 1\)-fructans were identified recently as Toll-like receptor (TLR) ligands \(^{(15)}\) and TLR2 is highly important in intestinal barrier incubation and fructan chain-length effects. Because \(\beta_2\rightarrow 1\)-fructans signal through TLR2, human embryonic kidney (HEK) human TLR2 (hTLR2) reporter cells were stimulated with ITF I–ITF IV.

**Investigational compounds.** To study the effects of \(\beta_2\rightarrow 1\)-fructans on intestinal epithelial cell barrier function, 4 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, and all fell below \(0.3 \times 10^{-3}\) endotoxin units \(\text{g}^{-1}\). Briefly, the mean chain length of these formulations is as ITF I (Frutalose OFP; Sensus) < ITF II (Frutafit CLR; Sensus) < ITF III (Frutafit HD; Sensus) < ITF IV (Frutafit TEX; Sensus). ITF I is a fructooligosaccharide compound, with chain lengths of \(\leq\)DP10. ITF II is an inulin, enriched with fructooligosaccharides, with most chains \(\leq\)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 ITF IV consist predominantly of chains ranging from DP10 to DP60. Chain-length profiles of the applied formulations are summarized in Figure 1.

**Materials and Methods**

**Experimental design.** T84 intestinal epithelial cells were grown to differentiation 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-mediated resistance, and these were used to calculate the AUC. To establish whether \(\beta_2\rightarrow 1\)-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 \(\beta_2\rightarrow 1\)-fructans protect T84 cells against PMA-induced loss of TEER and whether the \(\beta_2\rightarrow 1\)-fructan chain-length profile is important in TEER modulation. T84 cells were incubated for 24 h, with 4 different formulations of \(\beta_2\rightarrow 1\)-fructans of different mean degree of polymerization (DP) and DP profile (ITF I–ITF IV) provided by Sensus, followed by addition of PMA \((10 \text{ nM}; \text{Sigma-Aldrich Chemie})\). The second experiment was designed to investigate the possible time dependency of the effect of \(\beta_2\rightarrow 1\)-fructan incubation. Cells were stimulated in different orders with different compounds: 1) protocol I: incubation with PMA for 6 h, followed by removal of the medium and addition of \(\beta_2\rightarrow 1\)-fructans in culture medium at a final concentration of 100 mg/L; or 2) protocol II: incubation for 24 h with \(\beta_2\rightarrow 1\)-fructans, followed by addition of PMA. This stimulation medium was left on the cells for at least 6 h to allow the PMA to take effect. As the interval for recovery in protocol I, a fixed period of time was taken (12 h) after removal of PMA medium. This period was based on the mean 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, the AUC was plotted for this timeframe relative to the AUC of untreated controls (without PMA), which was set to 100%. For protocol II, the interval for protection against the reduction of TEER was based on the timeframe starting at the addition of PMA until the maximal decrease in TEER was reached (after 6 h), and this fixed timeframe was taken to calculate the AUC relative to untreated controls. With the third experiment, we aimed to study the role of TLR2 in \(\beta_2\rightarrow 1\)-fructan–mediated effects on T84 cells. To this end, T84 cells were incubated with culture medium or TLR2 blocking antibody (catalog no. pab-hlitr2, 5 mg/L; InvivoGen) for 10 min before the addition of \(\beta_2\rightarrow 1\)-fructans. Cells remained in this medium for 24 h, followed by addition of PMA. The 6 h timeframe after PMA addition was used for AUC calculations. Finally, to confirm that \(\beta_2\rightarrow 1\)-fructans signal through TLR2, human embryonic kidney (HEK) human TLR2 (hTLR2) reporter cells were stimulated with ITF I–ITF IV.

**TEER measurements.** Multiple electrode gold-plated 8-well chamber slides (8W10E; IBIDI via Applied Biophysics) were coated with...
400 μL/well of a 0.2% L-cysteine (Sigma-Aldrich Chemie) 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 1% PureCol bovine tail collagen (Nutacon) and 0.1% BSA (Sigma-Aldrich Chemie) in PBS. Wells were then washed twice with culture medium, and cells were seeded at a density of 2 × 10^4 cells per well in a final culture volume of 400 μL/well. Before stimulation, the cells were maintained in the wells for 14 d to reach a stable TEER. Medium was changed twice a week. The chamber slides were put into an electric cell substrate impedance sensing incubator (Z-Theta model; Applied Biophysics), and resistance was measured continuously at multiple frequencies (46).

**HEK hTLR2 reporter cell culture and reporter assay.** HEK hTLR2 reporter cells were cultured, and NF-κB activity was determined as described previously (15). As positive control, the additional TL2 agonist heat-killed *Listeria monocytogenes* was applied by adding 20 μL of solution to 180 μL of cell suspension (1 × 10^5 bacteria/mL; InvivoGen).

**Statistical analysis.** Statistical analysis was performed using Prism 5.0 software (GraphPad Software). D’Agostino and Pearson omnibus normality test was used to test for normal data distribution. Statistical significance levels were determined by 1-factor ANOVA and Tukey’s multiple comparison test or by Dunnett’s multiple comparison test to compare treatment with controls. Results are expressed as means ± SDs. *P* values < 0.05 were considered statistically significant.

**Results**

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 the β2→1-fructan chain-length profile is important in TEER modulation, T84 cells were incubated with 4 different formulations of β2→1-fructans of different mean DP and DP profile (ITF I–ITF IV). The AUC for the 6-h time period after PMA addition was plotted for the different ITF treatments, as a percentage of the AUC of untreated controls, which was set to 100% (Fig. 2). The damage model of PMA treatment induced a decrease in TEER, resulting in an AUC of 61.5 ± 5.8% (*P* < 0.001) compared with controls. Strikingly, 24 h of preincubation of T84 cells with 100 mg/L 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 ITF 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.

### Fig. 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.

The protective effect of short-chain β2→1-fructans against PMA-induced T84 barrier loss is time dependent. To establish whether timing of short-chain β2→1-fructan incubation is important in their functionality, 2 protocols for incubation were applied: 1) protocol I: preincubation of T84 cells with PMA for 6 h, followed by removal of the medium and addition of short-chain β2→1-fructans (ITF I) in culture medium; and 2) protocol II: preincubation of T84 cells for 24 h with ITF I, followed by addition of PMA (Fig. 3). Figure 3A shows an example representative of relative TEER values obtained with protocol I and the 12-h interval used to calculate the AUC, which was subsequently plotted in Figure 3B. Figure 3, A and B, shows that protocol I did not induce TEER recovery effects compared with PMA treatment. A representative example of relative TEER values obtained with protocol II and the 6-h interval used to calculate the AUC are plotted in Figure 3C. Here, a substantial protection was established, minimizing the decrease in TEER at 6 h 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 (Fig. 3D). Over the 6-h 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.

Blocking of TLR2 inhibits short-chain β2→1-fructan–mediated protection of TEER. Because β2→1-fructans were identified recently as TLR ligands (13) 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 before the 24-h incubation with short-chain β2→1-fructans, followed by PMA challenge (Fig. 4). Because the effects of longer-chain β2→1-fructans did not induce protective effects, ITF III and ITF IV were not included in the TLR2 blocking experiments. Figure 4, A and B, shows 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 6-h timeframe after PMA addition was plotted in Figure 4C, showing that pretreatment with TLR2 blocking antibody substantially 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.
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 was 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 (Fig. 5). Short-chain β2→1-fructans (ITF I) induced a 2.8 ± 1.2-fold induction (P < 0.05) of NF-κB/activator protein 1 activation compared with control, and with increasing mean fructan chain length, the

**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.

**FIGURE 4** Effects of blocking TLR2 on β2→1-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.
fructans conferred stronger activation, up to 5.9 ± 0.5-fold induction (P < 0.05) for the longest mean fructan chain formulation (ITF IV) compared with 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 the best of our 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 ITF II) but not for the longer-chain formulations (ITF III and ITF IV). This is in accordance with a previous study in our group, in which chain-length differences of the applied ITFs have different TLR activation patterns (15). In this study, the short-chain β2→1-fructans induced a more anti-inflammatory cytokine pattern compared with the longer-chain formulations, indicating chain length–dependent differences in downstream effects in immune cells. In addition, 2 studies by Ito et al. (50,51) performed in rats corroborated chain length–dependent effects on immune cells and intestinal barrier function in vivo. The studies by Ito et al. demonstrated that β2→1-fructans stimulated intestinal immune variables in a chain length–dependent manner (50) and that short-chain β2→1-fructans reduced translocation of endotoxins and bacteria in a trinitrobenzene sulfonic acid-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 24 h, 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 mg/L) was based on our previous results in peripheral blood mononuclear cells (15), in which this dose already induced substantial cell activation in the form of production of several cytokines during 24 h of stimulation. In addition, several studies showed 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 timeframe of preincubation with β2→1-fructans can be reduced compared with the 24 h preincubation protocol while retaining a protective effect remains to be studied. This timeframe may give an indication of 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 additional 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–dependent 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 was described previously 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 ITFs 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 cellular response different from 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

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barrier function is the subject of additional research in our laboratory.

As an analogue of the endogenous second-messenger diacylglycerol, PMA induces activation and translocation of protein kinase C (PKC), leading to elevated intracellular Ca²⁺ 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 readout for TLR2 activation in the HEK reporter cell line was analyzed by the production of secreted embryonic alkaline phosphatase during NF-κB activation. In intestinal epithelial cells, TLR2 activation was shown to act as an NF-κB inhibitor (43). Because PMA can also target NF-κB, downstream of PKC-β1 via stabilization of NF-κB inhibitor α (60,61), inhibition of NF-κB could be 1 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, partly inhibiting the cellular response to PMA. Previous studies with inulin showed 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 that 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 was linked previously 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. Because they also have immunomodulating effects and prebiotic effects, they form a promising category of dietary fibers with regard to intestinal health effects, and additional studies into their effects after ingestion on physiologic, cellular, and molecular levels are warranted.

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