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The influence of calcium on pectin's impact on TLR2 signalling

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High intake of dietary fibres and calcium has been correlated to a lower frequency of Western disease such as allergy, asthma and obesity. How the combined higher intake of dietary fibres and calcium reduces the incidence of these diseases is unknown. Dietary fibre pectin can interact with Toll-like receptor (TLR) 2 and calcium in a degree of methyl-esterification (DM)-dependent manner. Low DM pectins interact stronger with TLR2 than high DM pectins. Since low DM pectins are known to bind calcium strongly, we investigated how calcium influences the DM-dependent impact of pectins on TLR2 signalling. We tested TLR2 activating, inhibiting and binding properties of pectins with DM18, DM52 and DM69 under 0 mM, 1 mM and 10 mM calcium conditions. None of the pectins activated TLR2, but pectins inhibited TLR2. Under 0 mM calcium conditions, especially DM18 and DM52 strongly inhibited TLR2 and bound strongly to TLR2. Addition of 1 and 10 mM calcium to these pectins reduced TLR2 inhibition and TLR2 binding. Our study shows that calcium reduces inhibition of TLR2 by low and intermediate DM pectins, but calcium has lower impact on TLR2 inhibition by high DM pectins. Calcium may therefore beneficially influence the impact of pectin on TLR2 signalling and contribute to an improved intestinal barrier function. A combined higher intake of pectin and calcium may therefore contribute to a lower incidence of Western diseases.

1. Introduction

A higher prevalence of typical Western diseases, such as inflammatory bowel disease, obesity and colon cancer, has been observed in industrialized countries.^{1,2} Many studies suggest that this higher prevalence of Western diseases is

related to a reduced intake of specific dietary factors.³ In particular, lower intake of both dietary fibres and calcium have been correlated with higher prevalence of Western diseases.^{1,4} An interplay between dietary fibres and calcium may be beneficial for the host as it was demonstrated that a combined higher intake of dietary fibres and calcium reduces the risk of colorectal cancer even more than either of the individual compounds.⁵ However, underlying mechanisms of how a combined higher intake of dietary fibres and calcium may decrease the incidence of Western diseases is unknown.

It has been suggested that dietary fibres act on the intestinal immune system and thereby prevent the development of the Western diseases.⁶ Dietary fibres can modulate intestinal immunity through gut microbiota or by direct interaction with immune receptors.⁶ Recently it has become more evident that dietary fibres interact with Toll-like receptors (TLRs), which are specific pattern recognition receptors of the immune system.^{7–9} These immune receptors are expressed on intestinal epithelial cells and immune cells^{10,11} and recognize specific molecular patterns of pathogens, damage molecules from cells or nutrients.¹² Activation of these receptors may enhance intestinal barrier function and stimulate intestinal immune responses.¹⁰ In addition to TLR activation, inhibition of these receptors by dietary fibres may be beneficial to prevent aberrant inflammatory responses.⁸ Dietary fibres mostly interact with TLR2 and TLR4 which they can both activate or inhibit.⁹ Through interacting with these receptors, dietary fibres may stimulate the intestinal barrier function and contribute to the lower incidence of diseases.¹³

Pectin may be a candidate dietary fibre that in combination with calcium may decrease the risk of developing Western diseases, because pectins have the ability to interact with TLRs.¹⁴ However, as pectins are sensitive for calcium binding,¹⁵ the *in vivo* effect of combining the two is unknown and needs to be explored. Of the different TLRs, pectins mainly interact with TLR2.^{8,14} Both calcium binding and TLR2-interacting properties of pectins are strongly dependent on the homogalacturonan (HG) regions of pectins.^{8,15} HG regions of pectins

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contain a backbone of galacturonic acid (GalA) residues which can be methyl-esterified at the C6 carboxyl group. This is expressed as the degree of methyl-esterification (DM).¹⁶ Low DM pectins contain blocks of non-esterified GalA residues with negatively charged carboxyl groups,¹⁵ which can interact with positively charged amino acids of the TLR2 protein.⁸ Pectins with a lower DM have therefore a stronger ability to interact with TLR2 than pectins with a higher DM.⁸ In addition, the blocks of negatively charged non-esterified GalA residues can also interact with positively charged calcium ions.^{15,17} Methyl-esterified GalA residues cannot interact with calcium, because they lack this negative charge of the carboxyl group.^{15,17} Pectins with a low DM have therefore more tendency to interact with calcium ions than pectins with a high DM.¹⁸

Pectins influence TLR2 signalling in a DM-dependent manner, but it is unknown if calcium influences affects the ability of pectin to interact with TLRs. To understand how calcium influences the DM-dependent impact of pectins on TLR2 signalling, the TLR2 activating and TLR2 inhibiting properties of pectins with a different DM were determined under 0 mM, 1 mM or 10 mM calcium conditions. Furthermore, TLR2 binding properties of pectins was determined under different calcium conditions using a specific binding ELISA.

2. Materials & methods

2.1 Pectins

Three lemon homogalacturonan pectins with a different degree of methyl-esterification (DM) were used. Low DM (DM18) and intermediate DM (DM52) pectins were obtained from CPKelco (Lille Skensved, Denmark) and high DM pectin (DM69) was obtained from Andre Pectin (Andre Pectin Co. Ltd, Yantai, China). Molecular weight was determined by high performance size exclusion chromatography, monosaccharide content was determined by gas-liquid chromatography¹⁹ and the DM was determined by gas chromatography method as previously described.²⁰

2.2 Cell lines

To study the combined influence of calcium and pectins on signalling of Toll like receptor 2 (TLR2) we used the HEK-Blue™ reporter cell line of human TLR2. The different HEK-Blue cell line contains a construct of hTLR2 (Invivogen, Toulouse, France). This reporter cell line also expresses Soluble Embryonic Alkaline Phosphatase (SEAP). The SEAP reporter gene is placed under the control of a NF-κB and an AP-1 responsive promoter.^{8,21} Upon activation of the TLRs by pectins or a specific agonist, high levels of intracellular NF-κB will lead to secretion of SEAP which can be quantified by QUANTI-Blue (Invivogen). To isolate TLR2-HA proteins for the TLR2 capture ELISA, HEK 293/hTLR2-HA (Invivogen) was used. HEK-Blue™ cells and HEK 293/hTLR2-HA were cultured in DMEM culture media (Lonza, Basel Switzerland) containing 10% de-complemented Fetal Calf Serum, 50 U ml⁻¹ penicillin

Table 1 Selective antibiotics conditions

Cell line (Invivogen)	Selection antibiotics (Invivogen)
HEK-Blue hTLR2 293/hTLR2-HA cells	HEK-Blue 1× Blasticidin (50 µg ml ⁻¹)

(Sigma, St Louis, MO, USA), 50 µg ml⁻¹ streptomycin (Sigma), 100 µg ml⁻¹ normocin (Invivogen) according to the manufacturer's instructions. The cells were cultured for three passages before they were maintained in selection medium (Table 1). Cells were incubated at 37 °C and 5% CO₂.

2.3 Reporter cell assay

To study whether pectins can activate or inhibit TLR2, activation or inhibition assays were performed with pectins using HEK-Blue™ cells expressing human TLR2 (Invivogen). The HEK-Blue™ TLR2 cells were seeded in a 96 well plate in a volume of 180 µl at 2.8×10^5 cells per ml. After 24 hours of incubation, DMEM medium (1 mM calcium) was replaced with 180 µl medium containing pectins in a concentration of 2.0 mg ml⁻¹. Pectins were dissolved in DMEM medium containing 0 mM, 1 mM or 10 mM calcium concentrations. The 0 mM calcium condition was achieved by adding 2.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma) to the DMEM medium. The activation assay was performed by 24 hours incubation with pectins and controls. Pam3CSK4 (Invivogen) at a concentration of 10 ng ml⁻¹ was used as positive control and culture medium (0 mM, 1 mM or 10 mM calcium) was used as negative control. For the inhibition assay, cells were pre-incubated for 1 hour with pectins and subsequently stimulated with 10 ng ml⁻¹ Pam3CSK4 (Invivogen). After 24 hours of incubation, TLR activation was quantified by adding 20 µl of cell supernatant to 180 µl of QUANTIBLUE. After 1 hour incubation at 37 °C, NF-κB activation was quantified at 650 nm using a Versa Max ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). TLR activation was represented as fold-change compared to the negative control, whereas TLR inhibition was represented as fold-change compared to Pam3CSK4. Each experiment was performed at least eight times.

2.4 Protein immunoprecipitation and capture ELISA for binding of TLR2 to pectin

hTLR2-HA protein was isolated from HEK 293/hTLR2-HA cells (Invivogen) as previously described.⁸ HA-tagged proteins were immunoprecipitated using Pierce® anti-HA agarose (Thermo Scientific, Waltham, MA, USA). The elution step was performed using 50 µg ml⁻¹ HA peptide (Thermo Scientific) for 30 min at 30 °C. Zeba Spin Desalting Columns, 40 K MWCO (Thermo Scientific) were used to remove HA peptide from the samples. TLR2-HA protein concentration was quantified using the BCA protein assay kit (Thermo Scientific).

To confirm that TLR2 binds to specific types of pectins, a capture ELISA was performed.⁸ Experiments were performed with ELISA buffer containing 1 mM CaCl₂ and 150 mM NaCl

in 0.05 M Tris buffer at pH 8.2. For 1 mM calcium concentrations, pectins were dissolved in this ELISA buffer. For 0 mM calcium conditions, pectins were dissolved in ELISA buffer containing 2.5 mM EGTA. Further steps were performed with ELISA buffer containing 1 mM CaCl_2 . Isolated TLR2-HA was applied in a concentration of 10 μg per well. For each pectin, rat-anti pectin antibody LM20 (1 : 100; Plantprobes, Leeds, UK) was used to detect the amount of bound pectin. For each pectin, rat-anti pectin antibody LM20 (1 : 100; Plantprobes, Leeds, UK) was used as positive control for pectin binding, to confirm even pectin immobilization. Next, biotin-labelled goat anti rat secondary antibody (1 : 500; Southern Biotech, Birmingham, USA) was incubated for 1 hour. Streptavidin anti-HRP antibody (Cell Signalling, MA, USA) was incubated for 1 hour at 1 : 1000 dilution. After this, 100 μl TMB substrate (Cell signalling) was added and after 30 min, the reaction was stopped by 100 μl stop solution (Cell signalling). Plate readout was measured at 450 nm using a Versa Max ELISA plate reader (Molecular Devices). All steps were performed at 37 °C. Each experiment was performed at least five times.

2.5 Statistical analysis

Statistical analysis was performed with Graphpad prism version 8.4.1. Normal distribution was confirmed using the Kolmogorov-Smirnov test. Values are expressed as mean $\pm$ standard error (SEM). Statistical comparisons were performed using two-way ANOVA for parametrically distributed data. Post-testing between week 1 and week 4 was performed with Sidak post-test (* $p < 0.05$ was considered as statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3. Results & discussion

3.1 Chemical characterisation of the pectins

The three lemon pectins were characterized for the degree (percent) of methyl-esterification (DM), molecular weight and sugar composition (Table 2). The pectins showed only minor differences in molecular weight and sugar composition and mainly differed in the DM (DM18, DM52 and DM69).

3.2 Calcium influences TLR2 inhibition by pectins, but not TLR2 activation

The impact of pectins on TLR2 signalling was strongly dependent on the DM.^{8,14} It is however unknown how calcium

impacts the influence of pectins on TLR2 signalling. Therefore, we investigated how different calcium levels impact TLR2 activation and TLR2 inhibition. Pectins with a different DM were dissolved in culture medium containing three different calcium concentrations: 0 mM calcium (2.5 mM EGTA), 1 mM calcium and 10 mM calcium. TLR2 activation and TLR2 inhibition by pectins under the three calcium conditions was determined.

In the TLR2 activation assay we did not find any pectin induced enhancement of TLR2 signalling. This was independent of the calcium concentration applied (Fig. 1A). This was different in the TLR2 inhibition assay in which calcium had a strong influence in a concentration dependent manner (Fig. 1B). The influence of calcium was stronger for the low DM (DM18) and intermediate DM (DM52) pectins and to a lower extent for the high DM (DM69) pectin. Under 0 mM calcium conditions, TLR2 inhibition was strongest with DM18 pectin ($74.7 \pm 5.3\%$ vs. Pam3CSK4, $p < 0.0001$) and DM52 pectin ($78.6 \pm 17.5\%$ vs. Pam3CSK4, $p < 0.0001$), whereas TLR2 was less inhibited by DM69 pectin ($57.1 \pm 13.0\%$ vs. Pam3CSK4, $p < 0.0001$). TLR2 inhibition by the DM18 and DM52 pectins was significantly reduced under 1 mM (31.7% , $p < 0.0001$ and 29.9% , $p < 0.0001$, respectively) and 10 mM calcium conditions (33.7% , $p < 0.0001$ and 24.7% , $p < 0.001$, respectively) compared to 0 mM calcium conditions. DM69 pectin showed the strongest inhibition of TLR2 under 1 mM conditions ($67.5\% \pm 6.3$ vs. Pam3CSK4, $p < 0.0001$). Very high calcium conditions (10 mM) also reduced TLR2 inhibition by DM69 pectins compared to TLR2 inhibition under 1 mM calcium conditions (19.7% , $p < 0.001$). These results suggest that calcium strongly impacts TLR2 inhibition by pectins, but that the influence of calcium on pectin-TLR interaction is stronger for pectins with a low or intermediate DM.

The differences in the influence of calcium between the DM18 and DM52 pectins and DM69 pectin may be related to the presence of blockwise distributed non-esterified GalA residues and the tendency of these blocks of non-esterified GalA residues to interact with calcium. Blocks of at least 7–20 sequential non-esterified GalA residues can interact with calcium ions^{15,17,22} resulting in a three dimensional network formation of different pectin molecules. Pectins with a lower DM generally have more blockwise distributed GalA residues than pectins with an higher DM, because of the large number of non-esterified GalA residues.²³ Calcium may therefore have a stronger impact on the low DM pectins (DM18) and inter-

Table 2 Structural characteristics of pectins. Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample. Molecular weight (M_w) as measured by HPSEC. Monosaccharide content of rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc) and Uronic acid (UA) was determined by gas chromatography

Pectin	Origin	M_w (kDa)	Monosaccharide content (mol%)					Carbohydrate content (%)
			Rha	Ara	Gal	Glc	UA	
DM18	Lemon	53	0	0	3	1	95	73
DM52	Lemon	74	1	3	6	0	89	80
DM69	Lemon	81	1	2	8	1	87	83

Fig. 1 Activation and inhibition of TLR2 by pectins under different calcium concentrations. TLR2 activation (A) and TLR2 inhibition (B) by DM18 pectin, DM52 pectin and DM69 pectin in the concentrations 2 mg ml^{-1} under 0 mM, 1 mM calcium and 10 mM calcium concentrations. Values are expressed as mean $\pm$ standard error (SEM). The statistical differences between the different calcium conditions were quantified using the two-way ANOVA test. The statistical differences between the different calcium conditions were quantified using the two-way ANOVA test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

mediate DM (DM52) pectins than the high DM pectin (DM69). The very high levels of calcium may, however, also block the remaining non-esterified GalA regions of the DM69 pectin and prevent thereby TLR2-1 inhibition by DM69 under 10 mM calcium conditions. Together, these findings suggest that calcium strongly influences the TLR2 inhibition by pectins with a high number of blockwise distributed non-esterified GalA residues. Calcium may have a lower impact on TLR2 inhibition by higher DM pectins.

3.3 Calcium strongly impacts TLR2 binding to low DM pectins

To further confirm the influence of calcium on binding of TLR2 to pectins, we performed a capture ELISA that measures the direct binding of TLR2 to pectins under 0 mM calcium and 1 mM calcium conditions. We excluded the 10 mM condition in this assay, because no differences were observed on TLR2 inhibition between the different pectins under 10 mM conditions. As shown in Fig. 2, TLR2 bound stronger to pectins under 0 mM calcium conditions compared to 1 mM calcium conditions. The impact of calcium on TLR2 binding

to pectins was, however, dependent on the DM of pectins. TLR2 bound 33.8% stronger to DM18 under 0 mM calcium conditions compared to 1 mM calcium conditions ($p < 0.05$). DM52 pectin also showed a trend ($p = 0.07$) in stronger binding to TLR2 under 0 mM calcium conditions compared to 1 mM calcium conditions. There was no difference observed in TLR2 binding to DM69 under low and 1 mM calcium conditions. Together, these results clearly show that TLR2 binds stronger to low and intermediate DM pectins than high DM pectins and that calcium reduces TLR2 binding to pectins.

Previously it was suggested that the interaction between TLR2 and low DM pectins was established through electrostatic interactions between positively charged amino acids of TLR2 with negatively charged carboxyl groups of pectins.⁸ The current study confirmed a stronger binding of TLR2 to pectins with an increasing number of non-esterified GalA residues, in absence of calcium. However, TLR2 bound weaker to the pectins in presence of calcium. As calcium interacts with the negatively charged carboxyl groups on non-esterified GalA residues,^{15,17,22} it may be suggested that calcium dampens the electrostatic interaction between TLR2 and pectins.

In addition to their microbiota dependent effects, pectins may enhance the intestinal immune barrier by direct interactions with TLR2¹⁴ and prevent thereby the development of disease.¹³ The addition of calcium to pectins may enhance these direct effects of pectins on TLR2 by preserving adequate TLR2 signaling under healthy and diseased conditions. Under healthy conditions, TLR2 is expressed at low levels on epithelial cells and immune cells in the intestine.²⁴ A higher intake of TLR2 inhibiting pectins, such as low DM or intermediate DM pectins, may limit TLR2 signaling in the intestine. Calcium may preserve this TLR2 signaling by limiting pectin-induced TLR2 inhibition. A combined higher intake of pectins and calcium may therefore be beneficial to preserve TLR2 signaling in the intestine under healthy conditions. Once diseased conditions develop, however, TLR2 is highly expressed by intestinal immune cells which induce strong immune responses after TLR2 activation.^{25,26} Pectin induced inhibition of TLR2 can be beneficial to prevent the activation of aberrant

Fig. 2 TLR2 binding to pectins under different calcium concentrations. TLR2 binding to DM18 pectin, DM52 pectin and DM69 pectin in the concentrations 2 mg ml^{-1} under 0 mM and 1 mM calcium conditions. Values are expressed as mean $\pm$ standard error (SEM). The statistical differences between 0 mM and 1 mM calcium conditions were quantified using the two-way ANOVA test (* $p < 0.05$).

immune responses as we have shown in a previous study.⁸ Limited TLR2 signaling may be required, however, to restore intestinal barrier function under these conditions.²⁶ Low and intermediate DM pectins are strong TLR2 inhibitors, but increasing levels of calcium will limit TLR2 inhibition by these pectins (Fig. 1). Here high DM pectins may play an important role in TLR2 inhibition as calcium did not affect TLR2 inhibition by high DM pectins and allowed TLR2 signaling to a limited level (Fig. 1). The dose of calcium intake should not be too high (closer to 10 mM), because that will also limit TLR2 inhibition by high DM pectins. Taken together, our results suggest that a combined higher intake of calcium and of pectins may influence TLR2 signaling beneficially under healthy and diseases conditions. These direct effects on immune receptors together with the microbiota dependent effects of pectins may contribute the lower risk of developing diseases.

4. Conclusion

In the current study we showed that calcium influences the impact of pectins on TLR2 signalling, but these effects were dependent on the DM of pectins. Calcium reduced the inhibition of TLR2 by low DM (DM18) and intermediate DM (DM52) pectins, but calcium had lower impact on TLR2 inhibition by high DM (DM69) pectins. A combined higher intake of calcium and pectins may stimulate TLR2 signalling and thereby the intestinal barrier.¹⁴ Our findings may ultimately lead to the understanding of how a combined higher intake of dietary fibres and calcium may prevent the development of Western diseases.

Abbreviations

DM	Degree of methyl-esterification
DB	Degree of blockiness
GalA	Galacturonic acid
HEK	Human embryonic kidney
HG	Homogalacturonan
Nf- κ B	Nuclear factor kappa-light-chain enhancer of activated B cells
SEAP	Soluble embryonic alkaline phosphatase
TLR	Toll-like receptor
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid

Conflicts of interest

There are no conflicts to declare.

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