Research Article

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Apple extract induces increased epithelial resistance and claudin 4 expression in Caco-2 cells

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Abstract

BACKGROUND: The small intestinal epithelium functions both to absorb nutrients, and to provide a barrier between the outside, luminal, world and the human body. One of the passageways across the intestinal epithelium is paracellular diffusion, which is controlled by the properties of tight junction complexes. We used a differentiated Caco-2 monolayer as a model for small intestinal epithelium to study the effect of crude apple extracts on paracellular permeability.

RESULTS: Exposure of crude apple homogenate to the differentiated Caco-2 cells increased the paracellular resistance, determined as trans-epithelial electrical resistance (TEER). This increase was linearly related to the concentration of apple present. The TEER-enhancing effect of apple extract was due to factors mainly present in the cortex, and the induction was not inhibited by protein kinase inhibitors. Apple-induced resistance was accompanied by increased expression of several tight junction related genes, including claudin 4 (*CLDN4*).

CONCLUSION: Crude apple extract induces a higher paracellular resistance in differentiated Caco-2 cells. Future research will determine whether these results can be extrapolated to human small intestinal epithelia.

Keywords: apple; Caco-2; claudin 4; permeability; resistance; TEER

INTRODUCTION

The small intestinal epithelium forms a large interacting surface with the outside world. Its prime function is to absorb nutrients for energy and biosynthesis, combined with acting as a selective barrier to protect the human system. The epithelial barrier is composed of a monolayer which consists mainly of enterocytes interspersed with goblet cells and endocrine cells,[1] with the paracellular space between cells being sealed off by tight junctions.[2] Tight junctions thus form an important cellular structure that determines overall barrier properties of the epithelium. Transport of compounds through the tight junctions is driven by passive diffusion down an electrochemical gradient, and the selectivity is determined by tight-junction composition.[2]

Tight junctions are complex protein assemblies in which the three-dimensional structure and functionality of individual components are just beginning to be unravelled.[3] Claudins are hypothesised to form pores in the tight-junction complex, and in this way control the nature and flux of paracellular transport.[2,3] Changes in paracellular permeability are brought about by changes in phosphorylation, protein composition and membrane properties.[2] A compromised intestinal barrier function is suggested to be associated with the pathogenesis of a number of intestinal diseases and food allergies.[4–6] Allergic reactions to food products or food intolerance can be associated with increased paracellular permeability, which in turn can exacerbate the immune response due to passage of foreign peptides that would challenge the immune system.[5,7]

Caco-2 cells can resemble small intestinal epithelial cells and serve as a model system to study effects of single food compounds on barrier function. The fruit- and vegetable-derived food compound quercetin has been shown to increase the trans-epithelial electrical resistance (TEER) over a Caco-2 monolayer.[8,9] The increased TEER was correlated with higher abundance of claudin4 (cldn4) proteins, and the stimulation was inhibited by broad-spectrum protein kinase inhibitors.[8] Another group of compounds that have been shown to induce epithelial resistance in the Caco-2 model system are polyunsaturated fatty acids (PUFAs), which were able to alleviate interleukin-4 induced epithelial leakage.[10] In contrast, capsaicin has shown to reduce TEER,[11,12] although a reduction in TEER is more difficult to interpret since the reduction might also be caused by cell damage due to toxicity.[12]

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Although single compound-induced changes in TEER give insights in the cellular effects leading to the changed physiology, this is a highly reductionist approach to study the effects of food. Whole food products are a combination of various related and unrelated compounds that can interact and together provoke the bioactive effects, which can be different from the effects induced by the compounds in isolation.[13] Instead of studying isolated compounds, we studied the effect of crude food extracts on TEER. Some work has been done to study the effect of whole products, like extracts of ginger, sweet pepper, and edible burdock, which showed either no effect, or a decrease in TEER.[14] In this paper we describe the effect of crude apple extract on TEER and show its TEER-enhancing effects on Caco-2 cells.

MATERIALS AND METHODS

Cell culture

ATCC Caco-2 cells were grown on membranes of 33.6 mm^2 of ThinCert transwells, put in 24-well suspension culture plates. Cells were seeded with 150 µL of 1.3 × 10^9 cells L^{-1} and grown for 21 days at 5% CO_2, 37 °C, using Dubelco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Bleiswijk, The Netherlands) with 4.5 g L^{-1} glucose, 0.58 g L^{-1} glutamine, no pyruvate, supplemented with heat-inactivated (45 min 56 °C) fetal bovine serum (FBS; Hyclone PerBio, Etten-Leur, The Netherlands) to 9.1%. Cells reached confluence within 4 days of seeding. Cells were used with passage numbers 30 to 54; medium was replaced three times per week.

In vitro digestion

Jonagold apples were obtained from a local supermarket. Representative wedges of apple, without core were taken and 50% of weight 150 mmol L^{-1} NaCl–5 mmol L^{-1} KCl was added and mashed with a hand blender. Twenty grams of sample was put in a 50 mL tube, the pH was adjusted to 2 with HCl, and 0.667 mL of 40 g L^{-1} porcine pepsin added, after which the samples were incubated for 30 min at 37 °C. Sufficient 1 mol L^{-1} $NaHCO_3$ was added to raise pH to at least 5.8, followed by 0.95 mL of 4 g L^{-1} porcine pancreatin and 0.5 mL of a mixture of sodium taurocholate and sodium glycodeoxycholate (176 mmol L^{-1} of each). The sample was adjusted to pH 6.5 with $NaHCO_3$, the headspace was flushed with nitrogen and the sample was subsequently incubated for 1 h at 37 °C. The pH of the sample was adjusted to 7.5 with $NaHCO_3$ and the weight of the sample was adjusted to 30 g. Samples were centrifuged for 30 min at 3023 × *g* at 4 °C. The supernatant was taken, flushed with nitrogen and stored at −80 °C until use. Water or 150 mmol L^{-1} NaCl–5 mmol L^{-1} KCl was used instead of apple for preparing *in vitro* digestion controls.

Preparation of crude apple extract

Representative wedges of Jonagold apples, without core, were taken and 125% of weight 150 mmol L^{-1} NaCl–5 mmol L^{-1} KCl was added to obtain a similar dilution as the *in vitro* digested apple, and mashed with a hand blender. Samples were transferred to tubes and centrifuged for 30 min at 3023 × *g* at 4 °C. The supernatant was taken, flushed with nitrogen and stored at −80 °C until use.

Exposure

Samples were diluted 1 : 1 with DMEM + FBS. The pH was adjusted with sodium hydroxide if necessary, using phenol red in DMEM as indicator. The cell medium was replaced the day before the exposure experiments. TEER was measured using a MilliCell-ERS Ω-meter (Millipore, Molsheim, France), twice before sample addition. Medium was removed from the apical and basal compartments, and diluted samples were added to the apical compartment and fresh DMEM + FBS was added to the basal compartment. TEER was determined after various times of exposure.

Inhibitor studies

Inhibitors [1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7); 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride (ML-7); 3-[1-(dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride (GF 109 203X); staurosporine; and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM)] were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and 2000× stocks were prepared in DMSO (96% ethanol for H-7). Medium was removed from the apical compartment and replaced with 1 : 1 DMEM + FBS: 150 mmol L^{-1} NaCl–5 mmol L^{-1} KCl, with the respective inhibitor. After a 30 min incubation, medium from the apical and basal compartments was removed and the respective samples (apple only; apple + inhibitor; inhibitor only) was added to the apical compartment and fresh DMEM + FBS was added to the basal compartment.

Gene expression studies

RNA was extracted and cDNA was prepared as described by Vreeburg *et al*.[15] In brief, TriZol (Invitrogen, Bleiswijk, The Netherlands) extraction with 200 µL TriZol was followed with DNaseI (Sigma-Aldrich) treatment and RNeasy (Qiagen, Venlo, The Netherlands) clean-up. cDNA was synthesised with iScript (BioRad, Veenendaal, The Netherlands). Affymetrix HG-U133 microarrays were used with GenChip hybridisation kits, using *in vitro* digested apple and controls. Microarray data was normalised using gcRMA.[16] A subset of the total expression data was selected based on gene ontology, and statistics were applied to this subset.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed using ribosomal phosphoprotein P0 (RPLP0) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes, which have been validated for this purpose.[15] Primer sequences and PCR settings were as described by Vreeburg *et al*.[15]

Statistical analysis

Statistical significance was calculated with individual, two-sided *t*-tests, or with univariate analysis of variance with a Dunnett's post-hoc test,[17] using either MS-Excel, or SPSS (PASW Statistics, IBM, Amsterdam, The Netherlands). False discovery rate was determined according to Benjamini and Hochberg.[18] The effects of inhibitors were tested by comparing the apple-induced TEER increase in presence of the inhibitor with the apple-induced TEER increase in absence of the inhibitor.

RESULTS AND DISCUSSION

Effect of apple extract on Caco-2 barrier function

Crude apple extracts induced an increase in trans-epithelial electrical resistance (TEER) of the Caco-2 monolayer (Fig. 1a). The effect occurred within 1 h of exposure, after which no or little further increase in TEER was observed (Fig. 1) and lasted for at least 6 h (data not shown). Induction of TEER values by

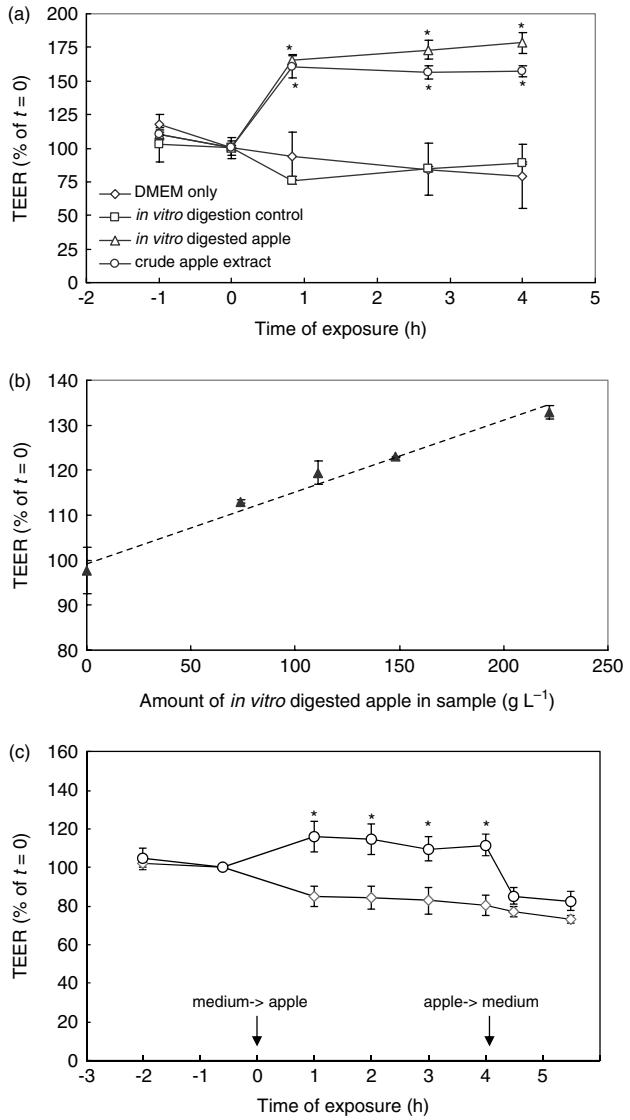

Figure 1. Modification of TEER by apple. (a) Change of TEER of Caco-2 cells after exposure to DMEM, *in vitro* digestion control (*in vitro* digested salt solution), crude apple extract, and *in vitro* digested apple and TEER measured over time. (b) Change in TEER upon 2 h exposure to different amounts of *in vitro* digested apple. A straight line is fitted through the data points. (c) Addition and removal of apple extract. Moments of sample addition and removal are indicated. Symbols are as given in panel (a). Averages ± SD, $n = 3$. *Indicates significant difference from control with $P < 0.05$.

apple extracts ranged from 20 to 90% of the original resistance, depending on the seeding batch of the Caco-2 cells (data not shown). Since food is subjected to various digestive steps before it reaches the small intestine, for which the Caco-2 cell layer is used as a model, we tested whether the induction of TEER by a crude apple homogenate remained after *in vitro* digestion. The *in vitro* digestion protocol included pH changes, enzymatic digestion with pepsin and pancreas enzymes, and addition of bile salts. *In vitro* digested apple showed a comparable TEER-enhancing effect as a crude extract (Fig. 1), indicating that the TEER enhancing activity can pass the human gastric environment.

TEER enhancing activity of apple extract is linearly related to the amount of *in vitro* digested apple as could be observed after exposures of the dilutions series (Fig. 1b). A concentration of 74 g

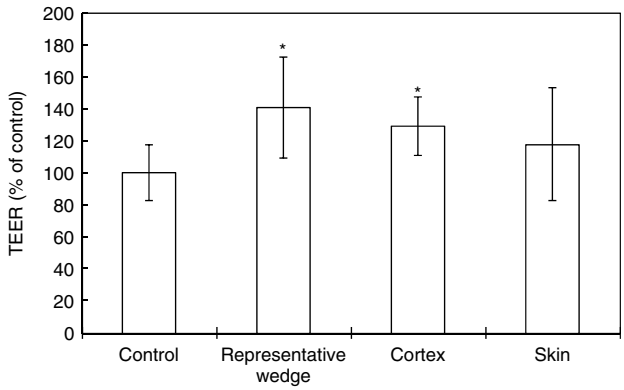

Figure 2. Effect of *in vitro* digested apple parts on Caco-2 TEER expressed as % relative to an *in vitro* digested salt control. *Indicates a statistical significant difference from control with $P < 0.05$. Averages ± SD, $n = 5$.

fresh weight L^{-1} apple already resulted in an increase of TEER. This concentration represents a dilution factor of more than 10, while a three- to five-fold dilution is expected in humans.[19] Assuming a similar dose–response range in Caco-2 cells as for human small epithelium, it is not unlikely that a similar response can be triggered in the human intestinal epithelium. However, experiments with humans using a dual sugar or Cr-EDTA uptake approach[20] will be needed to affirm the resistance altering effect of apple in humans.

We were interested to know whether the increase in epithelial resistance persisted after removal of the apple extract. When the apple-containing sample was removed after 4 h exposure, TEER returned to control levels within 30 min (Fig. 1c). Apple extract has thus the ability to induce an enhanced TEER in Caco-2 cells, but without a durable effect.

An apple can be roughly divided into three morphological parts: the skin, the cortex (flesh) and the apple core, of which the skin and the cortex are commonly used for consumption. To test whether the effect can be related to specific parts of the apple, skin and cortex tissue were tested separately. *In vitro* digested wedges of apple, containing cortex and skin, increased TEER of a Caco-2 monolayer, as did apple cortex alone (Fig. 2). However, *in vitro* digested apple skin, when added in equal amount to the cells did not result in a statistical significantly increase of the TEER (Fig. 2). The TEER-enhancing activity of apples is thus mainly situated in the cortex and to, a lesser extent, on a weight basis, in the skin. Since quercetin and quercetin glycosides have shown to enhance TEER in Caco-2 cells,[8] and Jonagold apples contain quercetin glycosides in the skin and not in the cortex,[21,22] we had expected to find most TEER-enhancing activity in the skin. These results therefore argue against quercetin glycosides being the active compound. The nature of the TEER-enhancing ingredient is still unknown.

Regulation of the apple-induced increase in TEER

Induction of TEER has been shown to be inhibited by the broad phosphokinase inhibitors H7 and staurosporin.[8] We tested the inhibiting activity of these inhibitors on apple-induced paracellular resistance together with other known kinase inhibitors and an intracellular calcium chelator (Table 1). None of the tested inhibitors blocked the apple-induced increase of TEER at the concentrations used (Fig. 3). The inhibitors have been applied to the cells at concentrations that are much higher than their reported dissociation constants,[23] or at concentrations reported to have an effect on TEER of Caco-2 monolayers.[24,25] ML-7 on its

Table 1. Phosphokinase inhibitors used for pharmacological study in TEER regulation

Compound	Type of inhibitor	Conc. used (μmol L^{-1})	Reference
H-7	Broad spectrum phosphokinase inhibitor	200	22
ML-7	Myosin light chain kinase inhibitor	30	22
GF 109203X	Phosphokinase A and C inhibitor	2	22
Staurosporine	Broad spectrum phosphokinase inhibitor	0.2	24
BAPTA-AM	Intracellular calcium chelator	10	23

H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine.
ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydro-chloride.
GF 1029230X, 3-[1-(dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride.
BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester).

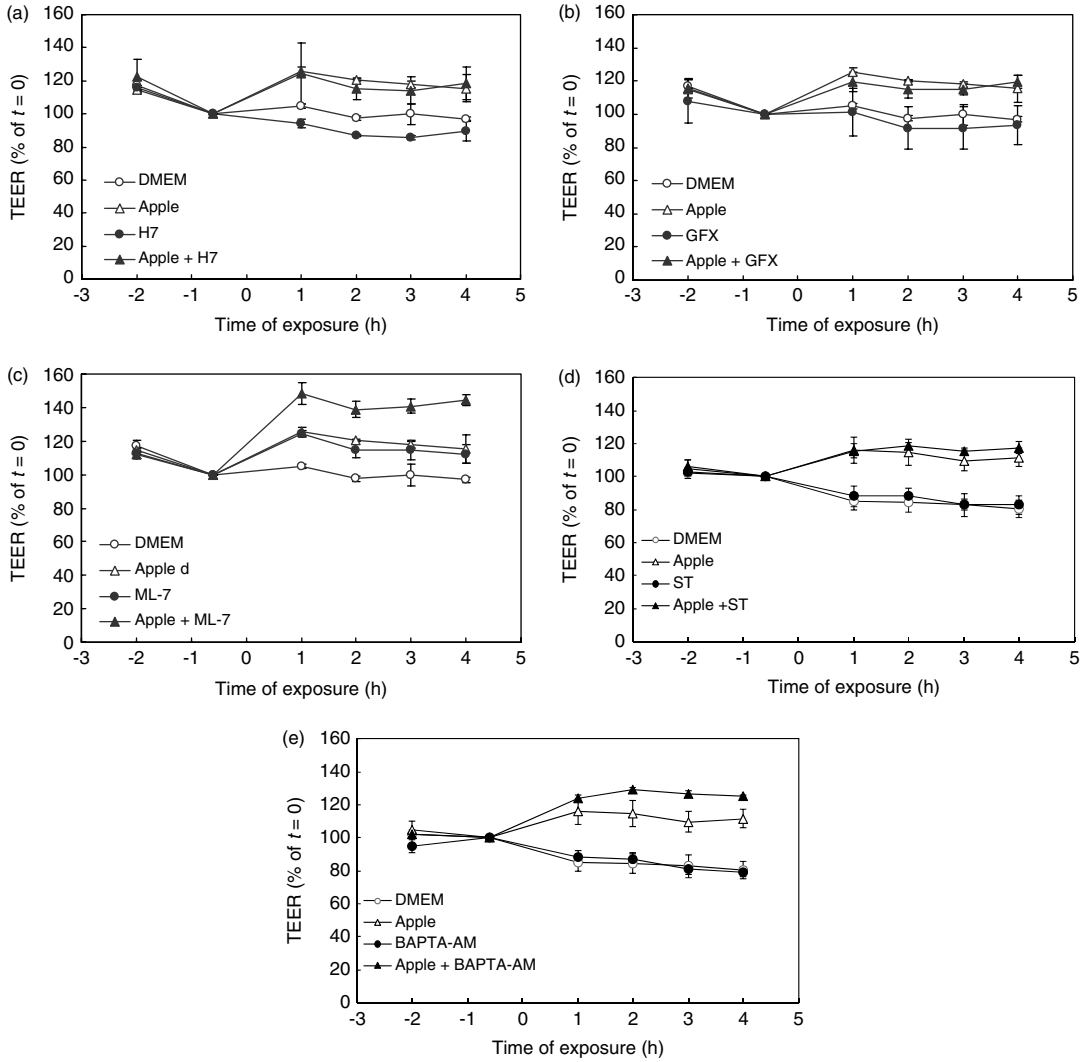

Figure 3. Effect of phosphokinase inhibitors and an intracellular calcium chelator on apple-induced TEER. (a) H7, (b) GF 109203X, (c) ML-7, (d) staurosporine, (e) BAPTA-AM. See Table 1 for abbreviations and concentrations used. Average ± SD, $n = 3$.

own enhanced the TEER of the Caco-2 monolayer, but addition of apple extract increased the TEER even more (Fig. 3c). An apple-induced increase in TEER was similar for cells treated with ML-7 as for cells without the myosin light chain kinase inhibitor (Fig. 3c). The apple-induced TEER enhancement was also not statistically different in the presence or absence of the intracellular calcium chelator BAPTA-AM (Fig. 3e). An apple-induced increase in paracellular resistance therefore did not involve phosphokinase A, phosphokinase C, phosphokinase G, myosin light chain kinase, or intracellular calcium signalling.

Effect of apple on tight-junction-related gene expression

Paracellular transport is regulated by tight junctions between the cells. In order to analyse the genes involved in the tight

Table 2. Genes that are ascribed to the tight junction cellular compartment (GO:0005923) and whose expression in Caco-2 cells was different after exposure to *in vitro* digested apple compared with *in vitro* digestion controls, with a false discovery rate <0.05

Gene symbol	Gene name	Fold induction*
ARHGAP17	Rho GTPase activating protein 17	1.13
ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	1.10
ASH1L	Ash1 (absent, small, or homeotic)-like (*Drosophila*)	0.92
CGN	Cingulin	0.90
CLDN1	Claudin 1	1.27
CLDN12	Claudin 12	1.09
CLDN16	Claudin 16	1.13
CLDN17	Claudin 17	1.17
CLDN2	Claudin 2	1.37
CLDN23	Claudin 23	1.27
CLDN4	Claudin 4	1.38
CLDN5	Claudin 5	0.93
CLDN7	Claudin 7	0.91
CRB3	Crumbs homolog 3 (*Drosophila*)	1.28
CXADR	Coxsackie virus and adenovirus receptor	1.06
F11R	F11 receptor	1.16
IGSF5	Immunoglobulin superfamily, member 5	0.88
INADL	InaD-like (*Drosophila*)	0.88
LIN7A	Lin-7 homolog A (*C. elegans*)	0.84
LIN7C	Lin-7 homolog C (*C. elegans*)	1.06
LOC283999	Hypothetical protein LOC283999	1.20
MAGI1	Membrane associated guanylate kinase, WW and PDZ domain containing 1	0.90
MAGI3	Membrane associated guanylate kinase, WW and PDZ domain containing 3	0.78
MICALL2	MICAL-like 2	0.56
MPDZ	Multiple PDZ domain protein	1.16
MPP5	Membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	1.16
MPP7	Membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	1.18
MTDH	Metadherin	0.94
PARD6A	Par-6 partitioning defective 6 homolog alpha (*C. elegans*)	1.15
PARD6B	Par-6 partitioning defective 6 homolog beta (*C. elegans*)	1.29
PARD6G	Par-6 partitioning defective 6 homolog gamma (*C. elegans*)	1.13
PRKCZ	Protein kinase C, zeta	0.82
SHROOM2	Shroom family member 2	1.13
TJAP1	Tight junction associated protein 1 (peripheral)	0.87
TJP1	Tight junction protein 1 (zona occludens 1)	1.20
TJP2	Tight junction protein 2 (zona occludens 2)	1.09
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33 kDa	1.07

* A value of 1 represents no change in expression relative to a non-apple exposed control.

junction a microarray experiment was performed. Cells were exposed to *in vitro* digested apple and genes that are ascribed to the tight junction cellular compartment, according to the gene ontology consortium (GO:0005923), were analysed. This group was represented by 67 genes on the array, of which 37 showed an altered expression in response exposure to apple. The list of 37 genes is given in Table 2, and includes several of the pore-forming claudin genes. Expression of *CLDN1* and *TJP2* (formerly named *ZO-2*) was increased by apple exposure (Table 2). The expression of these genes have been correlated with the fast TEER enhancing effect of quercetin.[9] *CLDN4* expression was induced 1.38-fold when determined by microarray (Table 2); *CLDN4* expression has been linked to long term TEER enhancement.[9] Claudin 4 expression in Madin–Darby canine kidney (MDCK) II cells have linked the expression of claudin 4 with reduced paracellular permeability for sodium.[26] Apple-induced expression of *CLDN4* in Caco-2 cells was validated by RT-qPCR in a separate experiment for both crude apple extract, and *in vitro* digestion apple, showing a 2-fold induction (Fig. 4). However, given the fast reduction in TEER upon removal of apple from the incubation mixture (Fig. 1c), it is unlikely that incorporation of claudin 4 proteins in the tight junction complex are the mechanisms by which the TEER is increased. Enhanced *CLDN4* expression could be a preparation for the incorporation of claudin 4 into tight junctions for the long-term adjustment of paracellular resistance, as proposed by Suzuki and Hara.[9]

CONCLUSION

Apple can induce an increase in paracellular resistance measured as TEER in Caco-2 cells. This TEER enhancing activity is maintained after *in vitro* digestion simulation, and is mainly localised in the cortex. The localisation of the TEER-enhancing factor in the cortex argues for an active compound other than quercetin glycosides, which are present in the skin only. Apple-induced

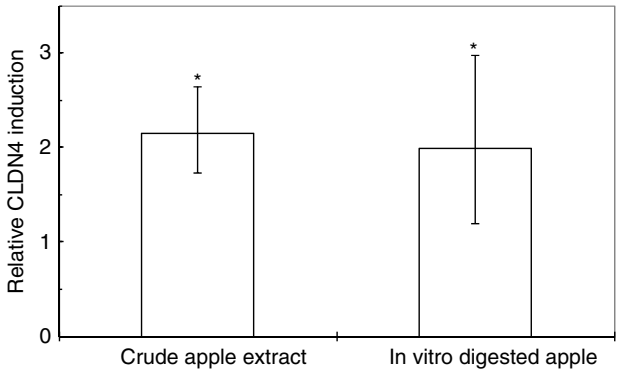

Figure 4. Effect of 4 h apple exposure on *CLDN4* expression. Expression values are corrected for expression of controls. Averages ± SD, $n = 3$. *Indicates significant difference from the control, with $P < 0.05$.

TEER enhancement is not inhibited by phosphokinase inhibitors and is correlated with increase *CLDN4* expression. These results indicate that food might modulate the barrier function of the intestine. Human trials will be needed to confirm these findings and establish a scientific base for dietary advice in cases where altered barrier function is desired.

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