Artificial sweeteners disrupt tight junctions and barrier function in the intestinal epithelium through activation of the sweet taste receptor, T1R3

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Running head: Sweet taste impacts intestinal barrier function
Word count: 4752 excluding abstract, legends and references
Number of figures: 6
Number of tables: 2

ABSTRACT

Breakdown of the intestinal epithelial barrier and a subsequent increase in intestinal permeability can lead to systemic inflammatory diseases and multiple-organ failure. Nutrition impacts the intestinal barrier, with dietary components such as gluten, increasing permeability. Artificial sweeteners are increasingly consumed by the general public in a range of foods and drinks. The sweet taste receptor (T1R3) is activated by artificial sweeteners and has been identified in the intestine to play a role in incretin release and glucose transport, however, T1R3 has not been previously linked to intestinal permeability.

The intestinal epithelial cell line, Caco-2, was used to study the effect of commonly-consumed artificial sweeteners, sucralose, aspartame and saccharin, on permeability. At high concentrations, aspartame and saccharin induce apoptosis and cell death in intestinal epithelial cells, while at low concentrations, sucralose and aspartame increase epithelial barrier permeability and down-regulate claudin 3 at the cell surface. T1R3 knockdown attenuates these effects of artificial sweeteners. Aspartame induces ROS production to cause permeability and claudin 3 internalisation, while, sweetener-induced permeability and oxidative stress is rescued by overexpression of claudin 3.

Taken together, our findings demonstrate that the artificial sweeteners sucralose, aspartame and saccharin, exert a range of negative effects on the intestinal epithelium through the sweet taste receptor T1R3.

INTRODUCTION

Under normal conditions, the intestinal epithelial barrier maintains selective gut permeability to allow nutrient absorption but provide a robust barrier and prevent the entry of pathogens and pathogenic molecules into the circulation. Disruption of the intestinal epithelial barrier results in a 'leaky gut', a key pathophysiological event seen in several chronic inflammatory disorders such as diabetes, pancreatitis, multiple organ failure and autoimmune diseases [1-3]. Impairment of the intestinal barrier and the subsequent increase in leak occurs predominantly in the distal small intestine and large intestine and can result in increased systemic inflammatory responses and tissue injury and, in some severe cases, sepsis and increased mortality [4]. There are a variety of treatments for impaired gut permeability, including prebiotics and metformin [5,6], however, there are limited large-scale clinical trials to establish the efficacy of interventions in reducing permeability, and the associated inflammation.

The intestinal barrier is maintained by two key mechanisms; (a) epithelial cell homeostasis to regulate cell numbers forming the barrier, and (b) homotypic junctional complexes to regulate paracellular permeability across the barrier [7]. Intestinal epithelial homeostasis is established by equilibrium between cell proliferation and cell death, with dysregulated or excessive epithelial cell death associated with diseases of impaired barrier integrity and leak across the paracellular space [4]. The paracellular space is largely modulated by tight junction (TJ) proteins which control the movement of water, nutrients and electrolytes across the epithelium into the interstitial fluid [8]. TJs are a family of around 50 proteins of which the main TJ proteins are occludins, claudins and junctional-adhesion molecules (JAMs) [9]. Despite this, knockout studies demonstrate a key role for claudins, rather than occludins and JAMs, in maintaining barrier integrity [10]. Claudins can form homotypic or heterotypic interactions at the epithelial cell-cell junction to form a seal and reduce leak, for example, claudins 1, 3, 4, 5, 8, 11 and 14, or form a pore to increase leak, for example, claudins 2, 7, 10 and 15 [9,11,12]. Expression of claudins at the TJ can be regulated via PKC-mediated phosphorylation and downstream intracellular trafficking pathways, such as reduced lysosomal degradation or increased trafficking to the TJ complex at the epithelial cell surface, to influence barrier integrity [13-16]. As such, diseases associated with a leaky gut, such as Crohn's disease and ulcerative colitis, have been demonstrated to impact expression of claudins in the small and large intestine, however, the mechanism for this is not clear [16,17]. Studies are therefore required to understand the mechanisms which regulate expression and function of claudins in the intestinal epithelium and how these proteins may be involved in controlling leak across the gut.

There is increasing evidence that consumption of a high-fat diet and excessive alcohol intake leads to intestinal permeability and metabolic endotoxemia [18-20]. These changes in permeability

are associated with increased oxidative stress and reorganisation of claudin expression at the tight junction [21,22]. While a link between the Western diet and intestinal permeability is established, the effect of food additives found in the diet is not well-understood. A range of artificial sweeteners are increasingly utilised as non-caloric sugar substitutes, of which aspartame, sucralose and saccharin are most commonly consumed in both food and beverages [23,24]. There is controversy regarding the metabolic effects of these acutely-sweet molecules, with studies demonstrating both a positive and negative role for artificial sweeteners in the diet [25-28]. Studies in humans and mice demonstrate that consumption of artificial sweeteners in the diet is linked with dysbiosis of the gut microbiota and an associated increase in levels of endotoxins secreted from these bacteria, such as lipopolysaccharide (LPS) [27,28]. LPS released from the gut microbiota is linked to an increase in intestinal permeability [29], however, while changes in the gut microbiota are a potential regulator of this permeability, the direct effect of artificial sweeteners on intestinal permeability is not well-understood.

In the studies presented here, we use the well-established intestinal epithelial cell line, Caco-2, to study the effect of the commonly-consumed artificial sweeteners, aspartame, sucralose and saccharin, on intestinal epithelial cell viability, monolayer permeability and claudin expression, and oxidative stress response. These studies utilised a variety of concentrations, which could be achieved in the diet, to address the impact of these sweeteners the intestinal epithelium. As artificial sweeteners stimulate the sweet taste receptors, T1R2 and T1R3, to elicit a taste response pathway [30], we also sought to demonstrate the role of these G-protein coupled receptors in regulating the effect of aspartame, sucralose and saccharin on the intestinal epithelium. We anticipate that findings from this study could expand our understanding of the physiological effect of artificial sweeteners and indicate the effect of dietary components on gut health.

MATERIALS AND METHODS

Cell lines and reagents

Human colon carcinoma cells (Caco-2) were purchased from Sigma-Aldrich (Dorset, UK), cultured in Eagle's Minimum Essential Media containing 10% fetal bovine serum, 1% penicillin/streptomycin and used between passage 35-50. siRNA and DharmaFECT™ reagent were obtained from Dharmacon (Cambridge, UK). Claudin 3 and control vector cDNA were purchased from GenScript (Piscataway, USA). Lipofectamine 3000™ reagent was purchased from Thermo Fisher Scientific (Paisley, UK). DCFDA and antibodies directed against claudin 2, 3, 4 and 7 were purchased from Abcam (Cambridge, UK), while, claudin 15 antibody was obtained from Novus Biologicals (Abingdon, UK) and T1R3 and actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V kit was purchased from BD Pharmingen (Wokingham, UK). All other reagents, including the artificial sweeteners saccharin, sucralose and aspartame, were purchased from Sigma Aldrich (Dorset, UK).

Animals and Ethics

The study used six male C57BL/6 mice bred at the Comparative Biology Unit at UCL's Royal Free Campus. Animals were allowed ad libitum access to water and a standard rat chow (Diet RM1, SDS Ltd, Witham, Essex, UK) until the time of experimentation. Animals were housed in groups of 3-4 and were maintained on 12-hour light-dark cycling (7AM-7PM) at a temperature of 22-5 ºC. At 8-10 weeks, mice were anaesthetized with an intraperitoneal injection of 60 mg.kg^{-1} pentobarbitone sodium (Pentoject, Animalcare Ltd, York, UK), and monitoring of the pedal and corneal reflex undertaken to ensure deep anaesthesia was achieved before the small and large intestine were removed. Euthanasia was performed by cervical dislocation, with death confirmed by cessation of the heartbeat. The gut was separated into the following sections: duodenum (beginning at the stomach and ending at the ligament of Treitz), jejunum (from the ligament of Treitz until halfway along the small intestine), ileum (remaining half of the small intestine until the caecum), proximal (1st half of the colon) and distal colon (2nd half of the colon). Each segment was flushed with ice cold 0.9% saline to remove any contents and transferred to a cold glass surface at 4°C and cut open. The mucosa was scraped off using a glass slide and placed in 1ml of RNAlater, snap frozen and stored at -80°C until use. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, Amendment Regulations 2012. The protocols were approved by the University College London (Royal Free Campus) Comparative Biology Unit Animal Welfare and Ethical Review Body (AWERB) committee.

RT-PCR

Total RNA was extracted from mucosal scrapes of the distinct regions of the mouse small and large intestine, or from Caco 2 cells, using Trizol reagent (Life Technologies, Paisley, UK) according to the manufacturer's instructions. RNA was DNase treated and reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). An RT-ve control was included for each sample. Claudin, taste receptor and β-actin transcripts were analyzed by real-time PCR using a Fast Start Essential Green Master kit (Roche Diagnostics, Mannheim, Germany) on a Roche LightCycler 96 (Roche Diagnostics, East Sussex, UK) using specific primers shown in Table 1 and 2. For all primers cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec. Relative gene expression compared to β-actin was established using the LightCycler 96 software.

Annexin V assay

Caco-2 cells were grown to 60% confluence in T-25 flasks prior to exposure with artificial sweeteners (range of concentrations) for 24 h. Artificial sweeteners were dissolved in the vehicle control (H_2O) and sterile-filtered to prepare a working stock solution. Adhered and floating cells were collected and incubated with binding buffer, annexin V and propidium iodide for 15 mins in the dark. Cells were then analysed with Accuri C6 Flow cytometer (BD Biosciences) and the percentage of positive cells for annexin V and propidium iodide were calculated with FlowJo (V10.2, Oregon, USA).

siRNA and cDNA transfections

Caco-2 cells were transiently transfected with siRNA specific to T1R3, or non-specific control siRNA, using DharmaFect™ 2 reagent as per manufacturer's guidelines. Alternatively. Caco-2 cells were transiently transfected with wild-type human claudin 3 cDNA (clone ID: OHu26411, vector: pcDNA3.1$^+$/C-(K)DYK), or DYK control vector cDNA) using Lipofectamine 3000™ as per manufacturer's guidelines. Cells were transfected at a seeding density of 0.5 x 10^4 cells per well of a 96 well plate, 2.5 x 10^4 cells per well of a Transwell insert, or 1.5 x 10^5 cells per well of a 6 well plate. Transfected cells were plated onto Transwell inserts or 96-well plates for analysis of permeability and whole-cell ELISA respectively. At 24 h post-transfection, cells were exposed to artificial sweetener (100 µM), or vehicle control (H_2O), in the presence and absence of the positive control LPS (1 µg/ml) for a further 24 h. Experiments were then performed as outlined in 'permeability' and 'whole-cell ELISA'. To confirm knockdown of T1R3 or overexpression of claudin 3, at 48 h post-transfection, cells were lysed with RIPA buffer, resuspended in Laemmli buffer and subjected to immunoblot analysis. Immunoblot analyses were performed on 10% SDS-PAGEs using a primary antibody specific to T1R3, DYK tag and β-actin at a dilution of 1:1000 and secondary antibody dilutions of 1:5000.

ROS assay

Caco-2 cells (1 x 10^4 cells per well) were plated on black-walled 96-well plate for 24 h followed by exposure to the cell permeant, fluorogenic dye 2',7' –dichlorofluorescin diacetate (DCFDA) (10 μM), or DMSO control, for 30 min at 37°C in the dark. DCFDA was then removed and replaced with artificial sweeteners (range of concentrations), or vehicle control (H_2O), or the positive control LPS (1 μg/ml) for 1.5 h. DCFDA fluorescence was measured at 488 nm using a fluorescent plate reader (Victor, Perkin Elmer) and measurements were compared to cells cultured in the absence of DCFDA.

Whole cell ELISA

Caco-2 cells (1 x 10^4 cells per well) were plated on black-walled 96-well plate for 24 h, followed by exposure to artificial sweeteners (range of concentrations), or vehicle control (H_2O), or the positive control LPS (1 μg/ml) for a further 24 h. Where stated, cells were first transfected with siRNA or also exposed to N-acetyl cysteine (NAC) (1 mM) or the vehicle for NAC (H_2O). Cells were then rinsed once with DPBS and fixed using 1% paraformaldehyde at room temperature for 10 min. Whole cell ELISA was then performed as previously described [31], in non-permeabilised Caco-2 cells, using antibodies specific to claudins 2 (ab76032), 3 (ab214487), 4 (ab210796), 7 (ab207300) and 15 (NBP1-59267). Antibodies are specific to αα 150 region of claudins [32]. Fluorescent-conjugated secondary antibodies were measured at 1 sec exposure time using a florescent plate reader (Victor, Perkin Elmer) and measurements from blank wells (no primary antibody) were subtracted to provide the data presented.

Epithelial monolayer permeability

Epithelial monolayer permeability was assessed using the FITC-dextran permeability assay and validated with TER (EVOM2; World Precision Instruments, Herts, UK). For analysis of monolayer permeability, Caco-2 cells were plated onto Transwell filters for 24 h, followed by exposure to artificial sweeteners (range of concentration), or vehicle control (H_2O), or the positive control LPS (1 μg.ml) for a further 24 h. Where stated, cells were first transfected with siRNA or also exposed to NAC (1 mM) or vehicle for NAC (H_2O). Permeability was measured by adding FITC-conjugated to 20 kDa dextran (FD20) to media in the upper chamber of the Transwell filter to a concentration of 5 μg/μl. FD20 was allowed to equilibrate for 180 sec at 37°C, and a sample (100 μl) of media from the lower chamber was collected and analysed at 488 nm using a fluorescent plate reader (Victor, Perkin Elmer). Permeability (%) was calculated by fluorescence accumulated in the lower chamber divided by fluorescence in the upper chamber, multiplied by 100.

Cell viability and morphology studies

Caco-2 cell viability was assessed using the Cell Counting Kit-8 (CCK-8). Cells were exposed to artificial sweeteners, or vehicle control (H_2O), for 24 h, followed by incubation with CCK-8 reagent for 2 h at 37°C. Absorbance was then assessed at 450 nm using a microplate reader (Tecan Sunrise) and viability was calculated as % normalised to vehicle.

Statistical analysis

The experimental number is presented in the legend for each experiment. *In vitro* experiments with Caco-2 cells were performed in duplicates. Data was analysed using GraphPad Prism 7.0. For two groups, the variance in data sets was analysed using the Mann-Whitney test followed by the T-test. For three or more groups, variance was assessed by using Bartlett's test with data sets not reaching significance studied by Kruskal-Wallis test followed by Dunn's test. For all other data sets, differences among the means were tested for significance in all experiments by ANOVA with Tukey's range significance difference test. Significance was reached when $p < 0.05$. Values are presented as mean ± standard error mean (S.E.M.).

RESULTS

High physiological concentrations of artificial sweeteners decrease viability and increase apoptosis of Caco2 cells through the sweet taste receptor (T1R3)

Artificial sweeteners stimulate the sweet-taste receptors, T1R2 and T1R3, which are G-protein-coupled-receptors [30]. We and others have demonstrated expression of T1R2 and T1R3 protein and mRNA in the intestinal epithelium where they have been identified to act as a sensor to stimulate glucose absorption and modulate incretin release [33-35]. Given the wide range of concentrations of different artificial sweeteners consumed in the diet [23], we sought to understand the dose-dependent effect of the commonly consumed artificial sweeteners sucralose, aspartame and saccharin on Caco-2 cell viability, apoptosis and cell death. Caco-2 cell viability was significantly decreased by aspartame and saccharin at concentrations ≥1000 µM (Figure 1a). Interestingly, there was a significant increase in cell viability following exposure to 1000 µM sucralose, however, at higher concentrations (10,000 µM), the sweeteners decreased cell viability (Figure 1a). Whilst there are limited studies to indicate the concentration of sweeteners found in the intestine, following consumption of artificial sweeteners, this range of concentrations (1-10 mM) is potentially achievable in the intestine, and thus physiologically-relevant, for members of the general population who regularly consume significant amounts of artificially sweetened foods. For example, a single chewing

gum contains 0.01 mM, one can of soft drink contains up to 2 mM of artificial sweetener and, more generally, the main additives in a range of products including diet drinks, sports drinks, snacks and confectionary are artificial sweeteners [23]. Given that the acceptable daily intake for these sweeteners is high (between 14-40 mg/kg body weight), it is likely that the public can consume high quantities of sweetener in the diet to achieve up to 10mM exposure to sweeteners [23]. Artificial sweeteners have been established to bind to the sweet taste receptor T1R2 and T1R3; therefore we next sought to establish the mRNA expression and cell surface protein levels of T1R2 and T1R3 in Caco-2 cells. Both mRNA(ratio: $1.48 \times 10^{-6} \pm 1.11 \times 10^{-7}$) and protein ($83.84 \pm 2.13$ r.f.u.)) expression of T1R3 was identified in untreated Caco-2 cells, however, T1R2 mRNA was not detected in the cells (undetected) and only low abundance of the protein was detected at the cell surface (13.69 ± 0.33 r.f.u.). We therefore next studied whether artificial sweeteners affected cell viability through T1R3, using siRNA knockdown of the sweet taste receptor ($63.5 \pm 2.7\%$ decrease, $p<0.05$, n=6 (Figure 1b). The significant decrease in cell viability following exposure to sucralose, saccharin and aspartame at 10,000 µM was abolished by T1R3 knockdown (Figure 1c). These findings were supported by studies with propidium iodide and Annexin V staining in Caco-2 cells exposed to 0 to 1000 µM artificial sweeteners, to measure cell death and apoptosis, respectively. A significant increase in cell death was observed at 1000 µM saccharin and aspartame (Figure 1d), matched by an increase in apoptosis at 10 and 100 µM (Figure 1e). As for cell viability studies, sucralose had no impact on Caco-2 cell death or apoptosis at concentrations ≤ 1000 µM (Figure 1d and e). These findings demonstrate that saccharin and aspartame induce apoptosis at lower concentrations (up to 100 µM) and cell death at higher concentrations (≤1000 µM). Taken together, these findings indicate that, at high but physiologically-relevant concentrations in the small intestine, artificial sweeteners sucralose, aspartame and saccharin decrease cell viability through binding to the sweet taste receptor, T1R3. The findings also demonstrate a differential effect of aspartame and saccharin versus sucralose on Caco-2 cell apoptosis and death.

Low physiological concentrations of artificial sweeteners sucralose and aspartame disrupt the intestinal epithelial barrier through the sweet taste receptor

Given the detrimental effect of high concentrations (≥ 1000 µM) of artificial sweeteners on Caco-2 cell viability, we next sought to establish the impact of a lower concentration (100 µM) on intestinal barrier function. The bacterial endotoxin, lipopolysaccharide (LPS), has been demonstrated to increase permeability of the intestinal epithelium [36]. Indeed, we demonstrate increased epithelial monolayer permeability when using both 1 and 10 µg/ml LPS using FITC-dextran permeability assay (% permeability - 1 µg/ml: $185 \pm 6.9\%$, $p<0.05$ versus vehicle; 10 µg/ml: $213.7 \pm$

10.7%, p<0.05 versus vehicle, n=6) and TER measurements (1 μg/ml: -263 ± 9.8 ohms, p<0.05 versus vehicle; 10 μg/ml: -304 ± 15.2 ohms, p<0.05 versus vehicle, n=6). Caco-2 cell viability, however, was only decreased at 10 μg/ml LPS exposure (% viability - 10 μg/ml: 70 ± 1.5%, p<0.05 versus vehicle, n=6)). Therefore 1 μg/ml LPS was used as a positive control for subsequent studies. Exposure of Caco-2 cells to the artificial sweeteners sucralose and aspartame significantly increased permeability of the epithelial barrier, to a similar level as seen for LPS (Figure 2a). Conversely, saccharin had no effect on epithelial barrier integrity (Figure 2a). Interestingly, siRNA knockdown of the sweet taste receptor, T1R3, attenuated sucralose- and aspartame-induced permeability, but had no impact on LPS-induced leak across the epithelial barrier (Figure 2b). These findings demonstrate the effect of artificial sweeteners, sucralose and aspartame, on intestinal epithelial barrier function.

Sucralose and aspartame modulate claudin 3 and 15 expression in intestinal epithelial cells through T1R3

Given the effect of sucralose and aspartame on epithelial barrier function, we next sought to establish the mechanisms regulating this process. Claudins are a key component of the tight junction complex and regulate epithelial barrier integrity. Although 26 claudins have been identified[37], in keeping with other studies [38,39] we demonstrate expression of claudins 2, 3, 4, 7, 8, 15 and 23 using RT-PCR analysis of the small and large intestine of mice (Figure 3a). From this data we chose the most abundant claudins and determined their expression levels in our Caco-2 cell model. Interestingly, claudin 4 was most abundantly expressed in Caco-2 cells, while claudin 7 was almost undetectable in the cells in comparison to the high levels present in the murine intestine (Figure 3b).

Claudins regulate permeability when expressed at the tight junction of the epithelial cell surface, with claudins 2 and 15 associated with pore-formation and leak, and claudins 3, 4 and 7 linked to tight junction sealing and reduced leak [37]. Therefore, we next studied the effect of sucralose and aspartame, on the cell surface protein expression of claudins, using LPS as a control to mimic breakdown of the epithelial barrier. Similar to mRNA expression, claudin 2 protein levels were found at low levels at the cell surface, with no significant effect of LPS on the protein (Figure 4a). In contrast, LPS exposure resulted in a significant decrease in claudin 3, 4 and 7 and a significant increase in claudin 15 protein expression at the cell surface (Figure 4b-e). Interestingly, both sucralose and aspartame significantly decreased claudin 3 surface expression and increased claudin 15 surface expression, similar to LPS exposure (Figure 4b and e), while the sweeteners had no effect on claudin 4 and 7 expression at the epithelial cell surface (Figure 4c and d). To assess the role of the sweet taste receptor, T1R3, in regulating the effect of sucralose and aspartame on claudin 3 and 15 expression, these experiments were repeated in Caco-2 cells with T1R3 siRNA. Knockdown of T1R3 levels

significantly abrogated the sweetener-induced decrease in claudin 3 surface expression but had no impact on the increase in claudin 15 levels (Figure 4f and g). Interestingly, the LPS-mediated decrease in claudin 3 was unaffected by T1R3 knockdown (Figure 4f).

Taken together, these data highlight the differential expression of claudins in the murine intestine and cultured intestinal epithelial cells, and demonstrates a key role for sucralose and aspartame, in regulating expression of claudin 3 and 15 at the epithelial cell surface. The data further indicates that sweeteners modulate claudin 3 and 15 expression at the tight junction through T1R3-dependent and T1R3-independent signalling pathways respectively.

Overexpression of claudin 3 rescues sweetener-induced barrier leak across the intestinal epithelium

To confirm that sucralose and aspartame regulate barrier leak across the intestinal epithelium through claudin 3, our next experiments were performed in Caco-2 cells overexpressing wild-type CLDN3-DYK cDNA, or the vector control (DYK). Western blotting and whole-cell ELISA confirmed overexpression of the construct at protein and cell surface levels, respectively (Figure 5a and b). Overexpression of claudin 3 had no impact on Caco-2 cell viability (Figure 5c), indicating no negative side effects of the transfection on the cells. Interestingly, leak across the intestinal epithelial cell monolayer, induced by aspartame and sucralose, was abrogated by claudin 3 overexpression (Figure 5d). These data demonstrate a key role for claudin 3 in regulating sweetener-induced permeability of the intestinal epithelium.

Aspartame, but not sucralose, increases oxidative stress in intestinal epithelial cells linked to barrier leak

Finally, we sought to establish the mechanism through which the sweet taste receptor and the artificial sweeteners sucralose and aspartame regulate claudin 3 expression at the cell surface. Oxidative stress is an important regulator of claudin 3 localisation in the intestinal epithelium and is linked to LPS-induced permeability [40,41], therefore we studied the effect of the artificial sweeteners, sucralose and aspartame, on the production of ROS in intestinal epithelial cells, using LPS as a positive control. Exposure of Caco-2 cells to LPS and aspartame, but not sucralose, significantly increased ROS production (Figure 6a). Interestingly, aspartame-induced ROS production was attenuated by exposure to the antioxidant NAC (Figure 6b) and knockdown of T1R3 (Figure 6c). We next studied the role that oxidative stress plays on sweetener-induced permeability of the Caco-2 cell monolayer and claudin 3 surface expression. Whilst NAC significantly attenuated aspartame-induced monolayer permeability, the antioxidant had no effect on sucralose-mediated leak (Figure 6d). Similarly, the reduction in claudin 3 expression at the cell surface, induced by aspartame, was

abrogated by NAC (Figure 6e), while sucralose-induced claudin 3 downregulation was unaffected by NAC (Figure 6e). Interestingly, aspartame-induced ROS production was significantly attenuated by overexpression of wild-type claudin 3 (Figure 6f) indicating a reciprocal relationship between oxidative stress and claudin 3.

Taken together, these data demonstrate that aspartame, but not sucralose, mediates claudin 3 expression at the tight junction and increases permeability of the epithelium through the production of ROS. The data further demonstrate a role for T1R3 in regulating aspartame-induced ROS accumulation in the intestinal epithelial cell.

DISCUSSION

At present, a large proportion of the population consumes artificial sweeteners, primarily aspartame, sucralose and saccharin [23]; however, there is significant controversy regarding the impact which artificial sweeteners in the diet exert on health. In particular, the effect of sweeteners on both the diversity and function of the gut microbiota, a key factor which regulates intestinal permeability, has been previously established with associated metabolic disruption linked to this dysbiosis [27,28]. However, whether there is a direct effect of these sweet-taste molecules on intestinal permeability is not well-understood. In the present study we demonstrate the effect of the artificial sweeteners, saccharin, sucralose and aspartame on intestinal epithelial cell claudin expression, barrier integrity and ROS production. Our studies show the detrimental and differential effects of these non-nutritive sweeteners at concentrations that would be typically found in the diet. Findings from this study also further our understanding of the mechanisms which regulate permeability of the intestinal epithelium and contribute to the controversy regarding the use of artificial sweeteners in the diet.

The intestinal epithelial barrier is vital in maintaining selective permeability between the small and large intestine, and circulation. The integrity of this barrier is maintained, in part, through cell survival, with an increase in epithelial cell apoptosis resulting in permeability, both *in vitro* and *in vivo* [42]. Tight junctions are another mechanism which regulates epithelial permeability, in particular the localisation of claudins in the tight junction complex. In this study, we identified claudin 3, 4, 7 and 15 expression in the murine small and large intestine and in Caco-2 cells, however, only claudin 3 and 15 are downregulated and upregulated, respectively, in response to sucralose and aspartame treatment. Previous studies have demonstrated that dietary components such as gluten alter claudin 3 and 15 expression [43,44], however, this is the first study to indicate that artificial sweeteners regulate these tight junction proteins. We further demonstrate the importance of claudin 3, rather than claudin 15, in regulating sweetener-induced permeability through T1R3. This may not be surprising given that

claudin 3 is a barrier-sealing tight junction protein which is down-regulated in settings of intestinal permeability, while the pore-forming claudin 15 is associated with mucosal differentiation in the small intestine [45,46]. Interestingly, our studies also demonstrate a role for claudin 3 in regulating aspartame-induced oxidative stress in the intestinal epithelial cell. Previous *in vivo* studies demonstrate a role for oxidative stress in dysregulation of claudin 1, 2 and 4 expression at the tight junction due to reduced levels of the antioxidant, superoxide dismutase, or increased levels of hypoxia-inducible factor-1 [47,48]. In addition, in gastric epithelial cells, claudin 3 was identified to be sensitive to oxidative stress, with siRNA knockdown of the tight junction protein exacerbating permeability of the monolayer [49]. Our studies indicate T1R3 as a key regulator of claudin 3-associated oxidative stress and monolayer permeability of the intestinal epithelial barrier.

Claudin expression is maintained through coordinated cell signalling processes in the intestinal epithelial cell. The shuttling of claudin proteins to the epithelial cell surface, to form the TJ complex, is dynamically regulated through intracellular trafficking processes [50]. Our studies demonstrate that the artificial sweeteners aspartame and sucralose bind T1R3 to cause reduced cell surface expression of claudin 3. Internalisation of claudin 3, associated with disruption of the TJ, has been observed to be caveolin- and flotillin-dependent [51,52]. It is therefore possible that downstream T1R3 signalling promotes these trafficking molecules to increase claudin 3 internalisation, however, further studies are needed to establish this mechanism. Furthermore, phosphorylation of TJ proteins, including claudin 3, by PKCζ has been demonstrated to play a key role in maintaining the TJ complex and therefore barrier function in the intestine [14,53]. Whilst the link between PKCζ and sweet taste sensing is not yet known, T1R3 stimulation by aspartame and sucralose may inhibit this PKC isoform and therefore promote disruption of the TJ. Finally, β-catenin, FOXO4 and hepatocyte nuclear factor alpha bind to claudin promoters to regulate expression of the TJ proteins, therefore sucralose and aspartame may affect claudin 3 levels by blocking these transcription factors to reduce expression in the intestinal epithelium [54-56]. Further studies are needed to understand the molecular mechanisms through which sweeteners reduce claudin 3 expression at the TJ, and the resulting downstream effects on barrier permeability and ROS production.

Expression of the sweet taste receptors, T1R2 and T1R3, has been established in the intestinal epithelium [26,33,35]. Similar to O'Brien and Corpe [57], data from the present study demonstrates expression of T1R3, but not T1R2, in Caco-2 cells. Artificial sweeteners have been demonstrated to bind to the sweet taste receptor in extra-oral locations to regulate a range of processes including glucose transport and insulin secretion [35,58]. In the present study, we demonstrate that the artificial sweeteners saccharin and aspartame exert a toxic effect on intestinal epithelial cells at high

concentrations while sucralose and aspartame increase epithelial permeability, and only aspartame causes oxidative stress. These studies are in contrast to previous findings where saccharin, but not aspartame or sucralose, was demonstrated to disrupt epithelial barrier integrity [59]. This difference in findings may be due to the short time point of 3.5 hour studied by Santos *et al* as oppose to the 24 hour time point assessed in the current studies. This time-dependent difference in tight junction formation and permeability has been observed in other epithelial tissue. In the choroidal plexus epithelium, a comparison of acute (3 hour) versus chronic (20 hour) exposure to the phorbol ester PMA demonstrates alternate effects on paracellular permeability [60]. This highlights the complex and dynamic nature of claudin organisation and barrier function of the epithelium and indicates that artificial sweeteners may have different impact on the intestinal epithelium in short and long term studies.

In vivo studies demonstrate that a saccharin-enriched diet causes accumulation of water in the stool of rats which may be indicative of leak across the intestinal epithelium [61]. Therefore whilst further *in vivo* studies are needed to demonstrate the direct effect of sucralose, saccharin and aspartame on leak across the intestinal epithelium, *in vivo* studies over a 3-day period match our *in vitro* findings at 24 hours. However, whilst sucralose and saccharin are resistant to hydrolysis in the small intestine, aspartame is rapidly hydrolysed into aspartic acid, methanol and phenylalanine [62-64]. Aminopeptidase A, the enzyme which is key for aspartame hydrolysis [62], is predominantly expressed and active in the mid and distal regions of the small intestine [65]. Proximal sections of the small intestine, the duodenum and early jejunum, are therefore likely be exposed to unmetabolized aspartame which is able to bind to the sweet taste receptor, T1R3. In contrast, latter regions of the small intestine, the late jejunum and ileum, are more likely to be exposed to the hydrolysis products of aspartame which do not bind to T1R3.

Our studies show that T1R3 is key to the observed cellular effects, with knockdown of the receptor attenuating the permeability, decreased viability and ROS production induced by sweeteners. While these findings need to be confirmed using an *in vivo* permeability model to establish the physiological relevance of consuming sweeteners at these concentrations, previous studies demonstrate that results from Caco-2 cell culture closely correlate with *in vivo* measurements of permeability [42]. The differential effects may be a result of altered intracellular signalling downstream of T1R3 for each artificial sweetener. These differences may be due, in part, to the structure of the artificial sweeteners, with the region of ligand binding in T1R3 mediating the signalling response. However, further studies are needed to identify the specific characteristics of each sweetener, their binding affinity to the sweet taste receptor, T1R3, and the resulting effect on intracellular signalling.

Artificial sweeteners are consumed by the general public at a range of concentrations, depending on the perceived sweet taste of the molecule and dietary choices, with one can of soft drink containing between 0.5-2 mM of sweetener [23]. A study of new food products launched in the USA between 1999 and 2004 shows that sucralose and aspartame are two of the most commonly-used artificial sweeteners, while saccharin is typically found in food and drinks blended with aspartame [23,66]. Our studies establish a detrimental effect of sucralose, saccharin and aspartame on cell viability at a concentration of 10 mM which, although high, is physiologically-achievable and within the acceptable daily intake given the increasing consumption of these sweeteners in both food and drinks [23,67]. Indeed, many studies in the field utilise concentrations up to 10 mM of artificial sweeteners (Santos; Mace; Malaisse WJ, 1998, 727, 10). Interestingly, at the significantly lower concentration of sucralose and aspartame (0.1 mM), we observed leak across the intestinal epithelial barrier *in vitro.* These findings indicate that consumption of both low and high amounts of these two sweeteners will disrupt the intestinal epithelium.

AUTHOR CONTRIBUTIONS

Acquisition of data: AS, BF, OO, ZG, HC, JM

Conception and design: HC, JM, AS,

Analysis and interpretation of data: HC, JM, AS, BF, OO, ZG

Drafting the manuscript for intellectual content: HC, JM

CONFLICT OF INTEREST DISCOSURE

The authors have nothing to disclose.

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TABLES

Primer	Catalogue number
T1R2	QT01026508
T2R3	QT00214270
claudin 2	QT0089481
claudin 3	QT00201376
claudin 4	QT00241073
claudin 7	QT00236061
claudin 15	QT00202048
β-actin	Forward TCACCCTGAAGTACCCCATC Reverse TAGCACAGCCTGGATAGCAA

Table 1: List of primers used for Caco-2 cell studies. Taste receptor and claudin primers were purchased from QIAGEN, while the actin primers were purchased from Sigma-Aldrich.

Gene	Nucleotide Accession number	Forward Primer	Reverse Primer	Reference
claudin 1	NM_016674	CCTTCGGGAGCTCAGGTGCG	CCGCGTTGGCCATGGCTCTT	[68]
claudin 2	NM_016675	TGGCGTCCAACTGGTGGGCT	ACCGCCGTCACAATGCTGGC	[68]
claudin 3	NM_009902	GGGCAGTCTCTGTGCGAGCC	CGGACGTCTGTCGCCGGGAA	[68]
claudin 4	BC132376	TGGGGACAGGCAAACCCGGA	CTTGCCGGCCGTAAGGAGCC	[68]
claudin 5	NM_013805	GCTCAGTGCACCACCTGCGT	GAACCAGCAGAGCGGCACGA	[68]
claudin 6	NM_018777	AGCACTCGCCCCCTCAACCTC	CATGGGCAGGGCACAGGACAC	[68]
claudin 7	NM_001193619	CACGCAGAGCACCGGCATGA	AGGGCGAGCACCGAGTCGTA	Blast
claudin 8	NM_018778	TCCCTGTCAGCTGGGTTGCCA	GCTCGCGCTTTAGGGCCACA	[68]
claudin 9	NM_020293	TCCCAAGTGGCACCTCACGGT	CGCGTTCCTCTCTGCTGGCTG	[38]
claudin 10a	NM_023878	GTGGCAGCAGGCAAGGCTGA	CACAGACGACGCTCGGGTGG	Blast
claudin 10b	NM_001160099	CTCCATCTCGGGCTGGGTGC	CAACGCCAGCATGGAGGGGA	Blast
claudin 11	NM_008770	TGGTTCCAGCTCGCCAACGC	TTACAGCACCTCGGCGGGCA	[38]
claudin 12	NM_001193659	AGGTATTCCCGAGCGGAGCCA	CCCGGAGGCTTCAGGGAACCA	[38]
claudin 13	NM_020504	TGACTCGTCCTGGTCCTGCCA	GGTCACCCTCCAAACGGGCA	[38]
claudin 14	NM_001165926	GCAGCTGCGGCAAAGGAGTCT	ACGGCCGTCTAATGGGTCCCT	[38]
claudin 15	NM_021719	TATGAACTGGGCCCCGCCCT	ATCCGAGGTGGCACGGGGTA	[38]
claudin 16	NM_053241	CCACGAACCAGGATGTGCCCG	GCGAGGGTCGTGGAGGTCAC	[38]
claudin 17	NM_181490	CTCCAGCGAGAGGGTCAAAG	AGCAGCAATATCCGCAGAGC	[38]
claudin 18	NM_001194921	CCTGACACCAGATGACAGCA	GGCAACATTTTGGCCAGAGG	[38]
claudin 19	NM_153105	CAGAGCCGGAGAGGGCGAACA	TCTGGGCAAGAGGGTTGCTGG	[38]
claudin 20	NM_001101560	GCACTCTAAAATACTCCATTC	TGAAGCAGACTCCTCCAGC	Blast
claudin 21		CTGGGACTATTGGGACTTCTG	AGGAGACTGGAAAGAGGGTAG	[69]
claudin 22	NM_029383	TTCCGAACGGCAACGCAGGC	CCCATCCCAGCAGGGAGAGCA	Blast
claudin 23	NM_027998	CGACGGACAGCATCGGCCTC	GGACTTGGGTGGCGGTCGTG	Blast
claudin 24	NM_001111318	GAACGGCCATGCAATCAGTAGGGC	DACGCAGGATTTCCAGAGCCCC	Blast
claudin 25	NM_171826	GAGAGGATGGGCGTATGCAG	ACTGCTCCAAGATGCTACGG	Blast
claudin 26	NM_029070	GTGCGGGTGGGATCGCGTAA	CCCACGCTCCCCGTCTGTTC	Blast
claudin 27	NM_001085535	TGGGTAGCCGGTGCCTCGAA	GCAGGCACCTAGCACAGGGG	[69]
β-actin	NM_007393	ATATCGCTGCGCTGGTCGTC	AGGATGGCGTGAGGGAGAGC	Blast

Table 2: List of primers used for murine intestine claudin expression studies. Primers sequences used were either published sequences or designed by Dr Rajagopal. The compiled list was kindly supply by Prof. A Yu of the University of Kansas Medical Center.

FIGURE LEGENDS

Figure 1: High physiological concentrations of artificial sweeteners decrease viability and increase apoptosis of Caco2 cells through the sweet taste receptor (T1R3). *Panel a:* Caco-2 cell viability was measured using the CCK8 assay following 24 hour exposure to the artificial sweeteners sucralose, saccharin and aspartame at concentrations ranging from 0.01 to 10,000 µM. Absorbance was normalised to 0 µM control and expressed as percentage viability. n=8. *Panel b and c:* Caco-2 cell viability was measured as for (a) following siRNA knockdown of T1R3 for 24 hours and exposure to sucralose, saccharin and aspartame (10 mM) for a further 24 hours. A representative blot of T1R3 and load control actin are shown to confirm siRNA knockdown using 50 µg protein (b). *Panel d and e:* Caco-2 cells were collected following exposure to sucralose, saccharin and aspartame at concentrations ranging from 0.1 to 1000 µM. for 24 hours and analysed by flow cytometry. Cell death (d) and apoptosis (e) were measured as propidium iodide and annexin V-positive and annexin V-positive cells respectively. n=5-6. Data is expressed as mean ± S.E.M. *$p < 0.05$ versus vehicle (0 µM).

Figure 2: Low physiological concentrations of artificial sweeteners sucralose and aspartame disrupt the intestinal epithelial barrier through the sweet taste receptor. *Panels a:* Permeability of the epithelial monolayer was measured, by FITC-dextran assay, following exposure to sucralose, saccharin and aspartame (0.1 mM) for 24 hours, using LPS (1 µg/ml) as a positive control. *Panel b:* Permeability of the Caco-2 cell monolayer was measured by FITC-dextran assay following siRNA knockdown of T1R3 for 24 hours and exposure to sucralose, saccharin and aspartame (0.1 mM) for a further 24 hours. % permeability was calculated normalised to vehicle treatment. n=6. Data is expressed as mean ± S.E.M. *$p < 0.05$ versus vehicle (0 µM).

Figure 3: Claudin mRNA expression profile in the intestinal epithelium *in vitro* and *in vivo*. Claudin mRNA transcripts were profiled in the small and large intestine and in cultured Caco-2 cells. *Panel a:* Three segments of the murine small intestine; the duodenum (stomach to ligament of Treitz), jejunum (ligament of Treitz until mid-small intestine), ileum (remaining half of small intestine until the caecum), and two segments of the large intestine; proximal (first half of the colon) and distal colon (second half of the colon); were collected for RT-PCR analysis. *Panel b:* Untreated Caco-2 cells were collected for RT-PCR analysis. The relative ratio is calculated as claudin compared to actin mRNA expression levels. Data is expressed as mean ± S.E.M of PCR reactions. n=6.

Figure 4: *Sucralose and aspartame modulate claudin 3 and 15 expression in intestinal epithelial cells through T1R3. Panels a-e:* Cell surface expression of claudins 2 (*panel a*), 3 (*panel b)*, 4 (*panel c)*, 7 *(panel d)* and 15 (*panel e)* protein was determined, with whole-cell indirect ELISA using chemiluminescence, in Caco-2 cells exposed to sucralose or aspartame (0.1 mM) or LPS (1 µg/ml) for 24 hours. *Panels f and g:* Cell surface expression of claudins 3 *(panel f)* and 15 *(panel g)* was determined in Caco-2 cells following siRNA knockdown of T1R3 for 24 hours and exposure to sucralose or aspartame (0.1 mM) for a further 24 hours. n=6. Data is expressed as mean ± S.E.M. *p<0.05 versus vehicle (0 µM).

Figure 5: *Overexpression of claudin 3 rescues sweetener-induced barrier leak across the intestinal epithelium.* Caco-2 cells were transiently transfected with cDNA encoding wild-type claudin 3 (CLDN3-DYK) or the vector control (DYK), or untransfected (UT) for 48 hour. *Panel a:* Total protein levels of claudin 3 in cells was measured by Western blot analysis of cell lysates using 50 µg protein. *Panel b:* Cell surface expression of claudin 3 was determined by whole-cell indirect ELISA using chemiluminescence in transfected Caco-2 cells. *Panel c:* Viability of transfected Caco-2 cells was assessed by CCK8 assay. Absorbance values were normalised to untransfected control and expressed as percentage viability. *Panel d:* Permeability of the transfected Caco-2 cell monolayer was measured, by FITC-dextran assay, following exposure to sucralose and aspartame (0.1 mM) for 24 hours. % permeability was calculated normalised to vehicle treatment. n=6. Data is expressed as mean ± S.E.M. *p<0.05 versus untransfected cells or vehicle (0 µM).

Figure 6: *Aspartame, but not sucralose, increases oxidative stress in intestinal epithelial cells linked to barrier leak. Panels a-c:* ROS production in Caco-2 cells was measured by fluorescence of DCFDA following exposure to sucralose or aspartame (0.1 mM) or LPS (1 µg/ml) as a positive control (*panel a)*, in the presence and absence of the anti-oxidant N-acetyl cysteine (NAC; 1 mM) (*panel b)* or following siRNA knockdown of T1R3 (*panel c)*. *Panel d:* Permeability of the Caco-2 cell monolayer was measured, by FITC-dextran assay, following exposure to sucralose and aspartame (0.1 mM) for 24 hours in the presence and absence of NAC. % permeability was calculated normalised to vehicle treatment. *Panel e:* Cell surface expression of claudin 3 was determined by whole-cell indirect ELISA using chemiluminescence in Caco-2 cells exposed to sucralose or aspartame (0.1 mM) in the presence and absence of NAC. *Panel f:* ROS production was measured by fluorescence of DCFDA in Caco-2 cells transiently transfected with CLDN3-DYK or control vector, exposed to aspartame (0.1 mM). % permeability was calculated normalised to vehicle treatment. ROS production was calculated as %

normalised to 0 µM. n=5-6. Data is expressed as mean ± S.E.M. *$p<0.05$ versus vehicle (0 µM); #$p<0.05$ versus vehicle for NAC.