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Specific Immunomodulatory and Secretory Activities of Stevioside and Steviol in Intestinal Cells

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Stevioside, isolated from *Stevia rebaudiana*, is a commercial sweetener. It was previously demonstrated that stevioside attenuates NF-κB-dependent TNF-α and IL-1β synthesis in LPS-stimulated monocytes. The present study examined the effects of stevioside and its metabolite, steviol, on human colon carcinoma cell lines. High concentrations of stevioside (2−5 mM) and steviol (0.2−0.8 mM) decreased cell viability in T84, Caco-2, and HT29 cells. Stevioside (2 mM) potentiated TNF-α-mediated IL-8 release in T84 cells. However, steviol (0.01−0.2 mM) significantly suppressed TNF-α-induced IL-8 release in all three cell lines. In T84 cells, steviol attenuated TNF-α-stimulated IκB → NF-κB signaling. Chloride transport was stimulated by steviol (0.1 mM) > stevioside (1 mM) at 30 min. Two biological effects of steviol in the colon are demonstrated for the first time: stimulation of Cl^- secretion and attenuation of TNF-α-stimulated IL-8 production. The immunomodulatory effects of steviol appear to involve NF-κB signaling. In contrast, at nontoxic concentrations stevioside affects only Cl^- secretion.

KEYWORDS: Stevioside; steviol; IL-8; immunomodulation; Cl^- secretion; intestinal epithelial cell lines

INTRODUCTION

Stevioside (SVS) is a natural noncaloric sweetener isolated from *Stevia rebaudiana* Bertoni, a perennial shrub of the Asteraceae (Compositae) native to Paraguay and Brazil. Stevioside, the main sweet component in the leaves, is approximately 300 times sweeter than sucrose on the basis of the organoleptic test (*1*). Extracts of *Stevia* leaves and its processed substances, including stevioside, have been used as a sugar substitute in a variety of foods, including beverages, confectionery, pickled vegetables, and sea foods in Japan, China, South Korea, and Taiwan, and stevioside has been approved as a dietary supplement in the United States (*2*). Stevioside has been suggested to exert beneficial effects on human health and is considered to be advantageous for patients suffering from obesity, diabetes mellitus, heart disease, and dental caries (*3*). It has been found to be antihypertensive (*4, 5*) and antihyperglycemic (*6*−*8*). In animal and cell culture models, stevioside has been reported to influence glucose metabolism (*9*) and renal function (*10*) and to have antioxidant (*11*), anti-inflammatory, and antitumor promoting (*12*) effects.

We have recently reported that in the human monocytic cells, THP-1, stevioside attenuates the action of LPS in stimulating the production of the inflammatory mediators TNF-α and IL-1β by interfering with the IKKβ → NF-κB signaling pathway (*13*). Interestingly, in the absence of LPS, stevioside alone is capable of causing a smaller increase in TNF-α secretion, an effect that appears to be partially mediated through the TLR4 receptor.

Despite its many biological effects, the mechanism by which stevioside enters the body is unclear. Stevioside is a diterpenic carboxylic alcohol with three glucose molecules (**Figure 1A**) and has a molecular weight of 804.9. It does not appear to be taken up across the intestinal mucosa when fed orally (*14*). The major metabolite of stevioside is the alcohol, steviol (SVO, **Figure 1B**), which has a molecular weight of 318.44 (*15*). Stevioside is not degraded into steviol by any of the enzymes of the mammalian, including human, digestive tract (*14, 16*) Yet, orally fed stevioside affects biological function. Thus, skeletal muscles, isolated from stevioside-fed rats, show increased glucose metabolism (*7*). Experiments with rats and hamsters suggest that stevioside is metabolized to steviol by the bacterial flora of the cecum (*17*). Likewise, bacteria isolated from the human colon, but not the small intestine, are able to transform stevioside into steviol in vitro (*14, 16, 17*). These authors concluded that only the bacteria from the cecum or colon were able to degrade stevioside into steviol. Recent studies in human volunteers further support this notion and demonstrate

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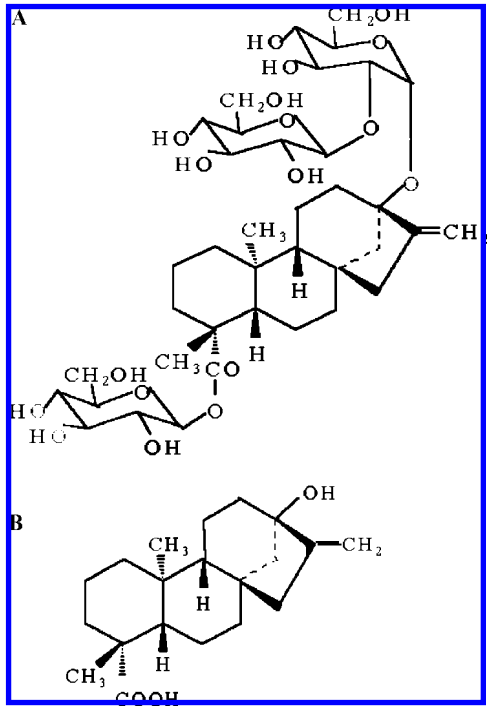

Figure 1. Structures of stevioside (**A**) and steviol (**B**).

that the major forms of excreted stevioside metabolites are steviol glucuronide and fecal steviol (*18, 19*). Other investigators demonstrated that colonic epithelial cell lines such as Caco-2 cells could take up steviol but not stevioside (*20*), suggesting the biological relevance of this metabolism.

Although studies have examined the effects of stevioside on glucose absorption in the mammalian small intestine, there have been no studies examining the effects of stevioside on biological function in the colon, a tissue where it is most likely metabolized in the intact animal. Therefore, the aim of the present study was to examine the effect of stevioside and its metabolite, steviol, on two major biological functions (immunomodulatory and secretory) in human colon carcinoma cell lines, T84, Caco-2, and HT29. All three cell lines are known to respond to the action of immunomodulators such as TNF-α and, in addition, T84 cells have been used extensively as a model of colonic Cl^- secretory activity. This study demonstrates for the first time a biological effect of steviol in the intestine, increasing Cl^- secretion and attenuating the ability of TNF-α to increase IL-8 production. In contrast, stevioside has an effect only on Cl^- secretion. The immunomodulatory effects of steviol appear to involve NF-κB signaling.

MATERIALS AND METHODS

Preparation of Stevioside and Steviol. Stevioside was extracted and purified (approximately 98% purity) from dried *S. rebaudiana* leaves as described by Adduci et al. (*21*). Steviol (approximately 90% purity) was obtained by oxidation of stevioside as described by Ogawa et al. (*22*). The purities of stevioside and steviol were determined by high-performance liquid chromatography conducted with a Waters model 510 liquid chromatograph (Waters, Millipore Corp., Milford, MA).

Cell Line and Tissue Culture Media. The T84 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in DMEM/F-12 medium containing 6% heat-inactivated newborn calf serum (Gibco BRL), penicillin (100 units/mL), and streptomycin (100 μg/mL). HT29 and Caco-2 cell lines (ATCC) were grown in DMEM/F-12 medium containing 20% heat-inactivated fetal bovine serum (Gibco BRL). The IEC-18 cell line (ATCC) was grown in DMEM containing 5% FBS (Gibco BRL), insulin (0.1 unit/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). These four cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT Assay. To detect the viability of cells, the method of Mosmann (*23*) using the MTT colorimetric assay was performed. In brief, T84, HT29, Caco-2, and IEC-18 cells were grown in 96-well plates until they reached confluence. Fresh medium was added, and the cells were further incubated for 8 h in a humidified atmosphere of 5% CO_2 at 37 °C with various concentrations of stevioside or steviol. At the end of 8 h, fresh medium and MTT solution (5 mg/mL) were added, and the cells were further incubated for 4 h in a humidified atmosphere of 5% CO_2 at 37 °C. A 200 μL aliquot of dimethyl sulfoxide (DMSO) was added, and the absorbance of each well was measured at 550 nm in a microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Determination of IL-8. T84, Caco-2, and HT29 cells were incubated with different concentrations of stevioside or steviol in the absence or presence of TNF-α (100 ng/mL, R&D Systems, Minneapolis, MN) for 8 h in a humidified atmosphere of 5% CO_2 at 37 °C. Supernatant fluids were collected and stored at −80 °C until IL-8 was assayed. Assays were performed using a commercial human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Western Blotting. T84 cells were stimulated for 45 min with various concentrations of steviol (0.1 and 0.01 mM) in the absence or presence of TNF-α (100 ng/mL). Cell lysates were extracted with RIPA lysis buffer (Santa Cruz, CA). The protein concentrations of the lysates were determined using the Bradford method (Bio-Rad, Hercules, CA). An equal amount of protein (30 μg) from each lysate was subjected to 12% SDS−polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF membrane. Membranes were incubated for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS) containing 1% Tween-20 (TBST). After washing with TBST, the membrane was treated with mouse anti-IκB-α antibody (Santa Cruz, CA; 1:500 dilution in TBST containing 1% non fat milk) or with mouse anti-NF-κB (p50) antibody (Santa Cruz; 1:500 dilution in TBST containing 1% nonfat milk) overnight at 4 °C and washed five times with TBST. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz; 1:10,000 dilution in TBST containing 1% nonfat milk) for 1 h, and the antigen−antibody complex was visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL) according to the manufacturer's recommendation.

Iodide Effluxes. Iodide efflux studies were performed according to the original method of Venglarik (*24*) and as modified by Chappe et al. (*25*). T84 cells were grown to 90% confluence in 35 mm^2 Petri dishes. Culture medium was removed, and cells were gently washed three times with 2 mL of iodide-free efflux buffer containing 136 mM sodium nitrate, 3 mM potassium nitrate, 2 mM calcium nitrate, 11 mM glucose, and 20 mM HEPES, pH 7.4. Cells were then incubated with iodide loading buffer (same as the efflux buffer except sodium iodide replaced sodium nitrate) for 1 h at room temperature in the dark. After incubation, extracellular iodide was removed by rapidly rinsing the cells three times with 3 mL of efflux buffer. Efflux buffer (2 mL) was then added to the dish, and the buffer was replaced with fresh efflux buffer after 1 min. Samples were thus collected at 1 min intervals for the duration of the experiment. For the initial three samples, the efflux buffer used was drug-free to establish a stable baseline. Subsequently, efflux buffer containing DMSO or agonists was added. To determine the iodide concentration in the collected samples, an iodide-sensitive electrode (Orion 96-53, Fisher Scientific) and a pH/mV-meter (Orion digital pH/mV 611) were used. Log concentrations of standards containing from 10^{-9}−10^{-1} M iodide were plotted against the mV measured at each of these concentrations to generate a calibration curve. The iodide concentrations of the test samples were then calculated on the basis of the calibration curve. At the end of the experiments, the cells were homogenized and sonicated in lysis buffer containing 1 mM EDTA, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM DTT, 25 mM Tris-HCl, pH 7.4, and 1 μg/mL each of leupeptin, pepstatin, and aprotinin. Total protein was measured using the Bradford method (Bio-

Rad). The cumulative iodide efflux curves show the total amount of iodide that was collected up to each time point and normalized to the protein in each dish.

Statistical Analysis. Data from at least three individual experiments were analyzed and presented as mean ± SEM. Statistical significance was determined using one-way ANOVA and Student−Newman−Keuls, with a value of $p < 0.05$ taken as being statistically significant.

RESULTS

Effect of Stevioside and Steviol on Cell Viability. Few studies have directly examined the effects of stevioside and steviol on cell viability. In one of the few such studies, concentrations of ≤1 mM stevioside and ≤0.1 mM steviol did not affect cell viability in THP-1 cells, as assessed by the MTT colorimetric assay (*13*). Most studies examining the effects of stevioside and steviol in cell lines have used concentrations of ≤1 and ≤0.1 mM, respectively. The only study using higher concentrations of stevioside examined the effects of 5 mM stevioside on glucose absorption in the everted gut sac assay, but tissue viability was not examined (*9*). Because the studies with THP-1 cells had demonstrated a partial (not complete) decrease in LPS-stimulated TNF-α release, we wished to try a wider range of concentrations to determine if stevioside could totally abrogate the effects of the immunomodulators. To do this, it was necessary to establish first whether the higher doses affected cell viability.

The MTT colorimetric assay was used to determine the effect of stevioside and steviol on cell viability of T84, HT-29, and Caco-2 cells, three cell lines derived from human colon carcinomas, and IEC-18, an immortalized rat small intestinal cell line. As shown in **Figure 2A**, stevioside, in doses ranging from 0.001 to 1 mM, had no cytotoxic effect in any of these cell lines. However, in all four cell lines it decreased cell viability to 76−82% at 2 mM and to 33−68% at 5 mM. The effects of steviol at >0.2 mM varied with cell type. As shown in **Figure 2B**, low doses of steviol (0.001−0.1 mM) had no cytotoxic effect in any of the cell lines examined. Whereas 0.2 and 0.8 mM steviol decreased viability to a similar extent in the four cell types (0.2 mM, to 80−90%; and 0.8 mM, to 7−34%), the effects of 0.4 and 0.6 mM were not uniform across cell types. Thus, as shown in **Figure 2B**, at these concentrations Caco-2 and HT29 cells showed 60−80% viability, whereas T84 and IEC-18 cells showed much lower (16−38%) viability. Similar effects on cell viability were seen at 3 and 6 h of the treatment.

Dose and Time Course of TNF-α on IL-8 Release. In THP-1 monocytes, LPS-stimulated TNF-α and IL-1β are decreased by stevioside, whereas steviol had no effect (*13*). To determine if stevioside and steviol affected immunomodulator release in colonic cell lines, IL-8 release was examined. This neutrophil chemoattractant is a major proinflammatory cytokine in colonic cells, and it forms the first line of defense in the gut. IL-8 can be expected to have a major impact on neighboring intraepithelial cells, on lymphocytes, and on macrophages. Because there are species differences in IL-8, our studies focused on the human cell lines, T84, Caco-2, and HT29. First, it was determined if IL-8 release was best measured under conditions of LPS or TNF-α stimulation. Whereas the former has the advantage of being more extensively studied in colonic cell lines, preliminary experiments indicated that 24 h of LPS treatment was needed to demonstrate a maximal effect on IL-8 release. As compared to the IL-8 secretion caused by TNF-α, 8 h of LPS treatment elicited only 16, 20, and 40% IL-8 increases in Caco-2, T84, and HT29 cells, respectively. In contrast, in all three colon carcinoma cell lines, TNF-α caused maximal increases in IL-8 release within 6−8 h. Therefore, the ability of stevioside and steviol to modulate TNF-α-mediated IL-8 release was determined.

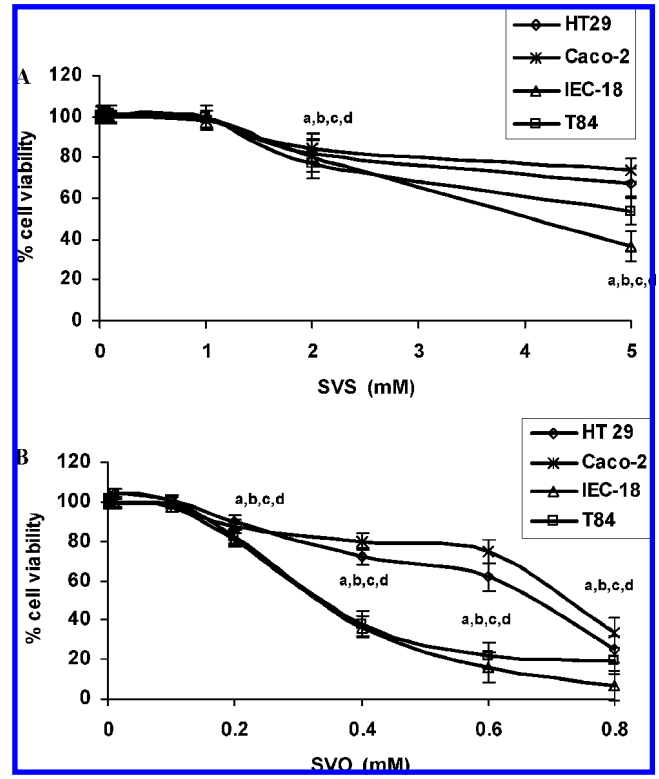

Figure 2. Effect of stevioside (**A**) and steviol (**B**) on cell viability in T84, Caco2, HT29, and IEC-18 cells. Cell viability was determined by the MTT assay (see Materials and Methods). Data are expressed as the mean ± SEM of three independent experiments. (a−d) Statistically significant difference in T84 (a), Caco-2 (b), HT29 (c), and IEC-18 (d) cell viability ($p < 0.05$) as compared with control in each cell line.

As shown in **Figure 3**, panels **A** and **B**, respectively, TNF-α caused a dose- and time-dependent increase in IL-8 release in T84, Caco-2, and HT29 cells. The highest concentration of TNF-α tested was 100 ng/mL, a dose 10 times higher than that previously used to demonstrate IL-8 response in T84 cells (*26*) and a dose used in other intestinal cell lines (*27*). At 100 ng/mL, TNF-α elicited significant release of IL-8, with the releases at 8 and 24 h being similar (**Figure 3B**). In all subsequent studies cells were exposed to 100 ng/mL of TNF-α for 8 h.

Effect of Stevioside and Steviol on IL-8 Release. As shown in **Figure 4A**, whereas low concentrations of stevioside had no effect on IL-8 release either ± TNF-α, 5 mM stevioside alone caused a slight increase in IL-8 release (571 ± 62 vs 183 ± 20 pg/mL in control) in T84 cells. In contrast, in Caco-2 and HT29 cells, stevioside even at 0.1−2 mM concentrations caused a significant release in IL-8 (**Figures 5A** and **6A**). Furthermore, in the presence of TNF-α, 2 mM stevioside potentiated TNF-α-dependent IL-8 release from T84 cells by an additional 35.6% (2573 ± 255 vs 1897 ± 193 pg/mL). In Caco-2 and HT29 cells, stevioside, in the presence of TNF-α, similarly caused an additional 500 pg/mL release of IL-8. However, these were not statistically significant, because the TNF-α-dependent IL-8 releases from Caco-2 and HT29 cells were almost twice the amount released by T84 cells (**Figure 4A** vs **Figures 5A** and **6A**). It is noteworthy that 2 mM stevioside causes a small but significant decrease in cell viability to approximately 80% in all three cell lines.

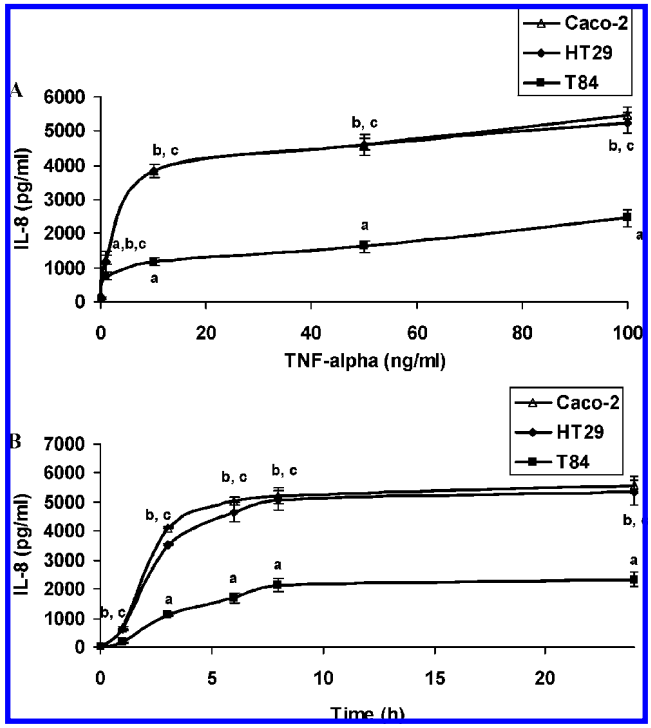

Figure 3. Effect of TNF-α on IL-8 release in T84 (a), Caco-2 (b), and HT29 (c) cells: dose response (**A**) and time course (**B**). Data are expressed as the mean ± SEM of three independent experiments. (a−c) Statistically significant difference in cytokine release ($p < 0.05$), as compared with control in each cell line.

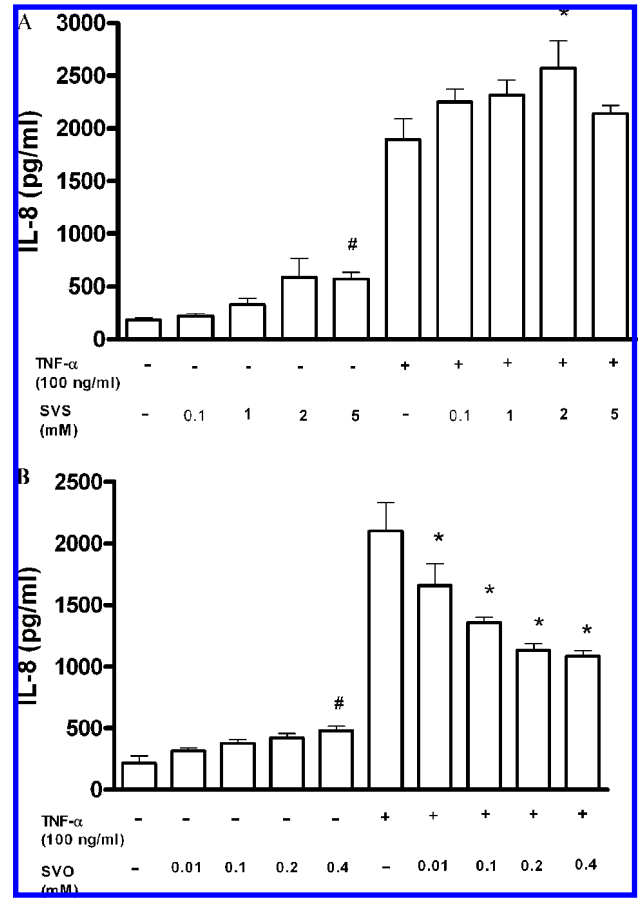

Figure 4. Effects of stevioside (**A**) and steviol (**B**) on the production of IL-8 in T84 cells. Cells were treated for 8 h in the presence and absence of TNF-α as indicated. Data are expressed as the mean ± SEM of three independent experiments. (∗, #) Statistically significant difference in cytokine release ($p < 0.05$), as compared with TNF-α-treated and untreated group, respectively.

As shown in **Figure 4B**, steviol (0.4 mM) caused a slight increase in IL-8 release (483 ± 33 vs 218 ± 57 pg/mL in control) from T84 cells, whereas it has no effect on Caco-2 and HT29 cells (**Figures 5B** and **6B**). However, in the presence of TNF-α, steviol (0.01, 0.1 mM) inhibited IL-8 release up to 21.1 and 35.4% (1659 ± 176 and 1358 ± 46 vs 2103 ± 228 pg/mL with TNF-α alone) in T84 cells (**Figure 4B**), 8.9 and 16.2% (4611 ± 181 and 4239 ± 130 vs 5065 ± 339 pg/mL with TNF-α alone) in Caco-2 cells (**Figure 5B**), and 9.1 and 17% (4567 ± 130 and 4171 ± 209 vs 5025 ± 332 pg/mL with TNF-α alone) in HT29 cells (**Figure 6B**). Equally important at these concentrations, steviol does not have any cytotoxic effects (**Figure 2B**). At 0.2 mM, steviol inhibited TNF-α-mediated IL-8 release to 46, 20, and 18% in T84, Caco-2, and HT29 cells, respectively. However, at this concentration steviol causes a small, 10−20%, decrease in cell viability (**Figure 2B**).

Interestingly, TNF-α had no effect on cell viability either in the presence or in the absence of stevioside or steviol. In other words, after 8 h of exposure to 100 ng/mL TNF-α, the cells were 100% viable and the decreases in cell viability seen at higher concentrations of stevioside and steviol are not altered by TNF-α.

Effect of Steviol on IκB-α → NF-κB Signaling Pathway. TNF-α activation involves the IκB → NF-κB signaling pathway with ligand activation resulting in the rapid phosphorylation of IκBs, disruption of the IκB/NF-κB complex, and subsequent rapid translocation of NF-κB to the nucleus (*28*). We examined whether this cascade is involved in the inhibitory effect of steviol on cytokine release using T84 cells, as steviol had the most robust effects in this cell line. In preliminary time course experiments (15, 30, 45, and 60 min), the optimal time for changes in IκB-α and NF-κB expression was found to be at 45 min of exposure to TNF-α (100 ng/mL). As expected, TNF-α decreased the expression of IκB-α protein when compared to untreated control samples in immunoblots using an IκB-α-specific antibody. This decrease was partially reversed when the cells were incubated in TNF-α + 0.1 mM steviol (**Figure 7A**). At 0.01 mM, although there is a trend to reverse the inhibition, the values are not statistically different (**Figure 7A**). At either dose, steviol alone had no significant effects on IκB-α expression. As shown in **Figure 7B**, the inhibitory action of steviol on TNF-α-treated T84 cells also affects NF-κB activation. TNF-α stimulated the expression of NF-κB (p50) protein, and this was significantly decreased in the presence of 0.1 mM steviol. Taken together, these data demonstrate that the IκB-α → NF-κB signaling pathway is involved in the action of steviol.

Effect of Stevioside and Steviol on Iodide Effluxes. To determine if stevioside and steviol had any effects on other cell functions in intestinal cells, their effect on perturbing the Cl^- secretory pathway in T84 cells was examined. To assess cellular Cl^- transport, we employed the iodide efflux assay using an iodide selective electrode, a convenient procedure that circumvents the use of radioisotopes. Iodide effluxes from T84 cells treated with 0.2% DMSO (control), a cAMP cocktail [8-Br-cAMP (100 μM) + forskolin (10 μM) + IBMX (100 μM)], stevioside (1 mM), and steviol (0.1 mM) were compared. As shown in **Figure 8**, at 3, 5, and 10 min, the cumulative iodide efflux in control was 49.9 ± 14.1, 66.5 ± 7, and 99.1 ± 14.5

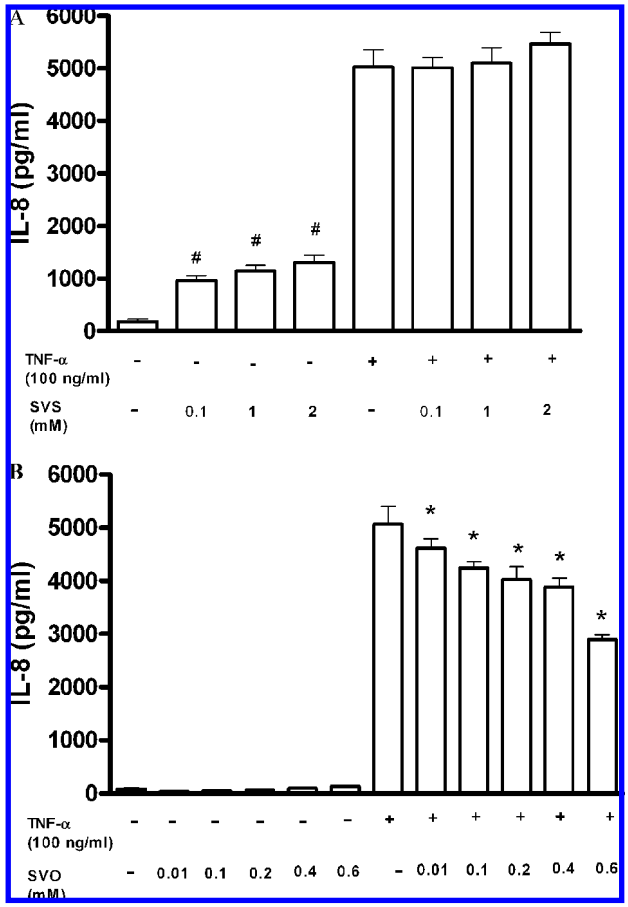

Figure 5. Effects of stevioside (**A**) and steviol (**B**) on the production of IL-8 in Caco-2 cells. Cells were treated for 8 h in the presence and absence of TNF-α as indicated. Data are expressed as the mean ± SEM of three independent experiments. (∗, #) Statistically significant difference in cytokine release ($p < 0.05$), as compared with TNF-α-treated and untreated group, respectively.

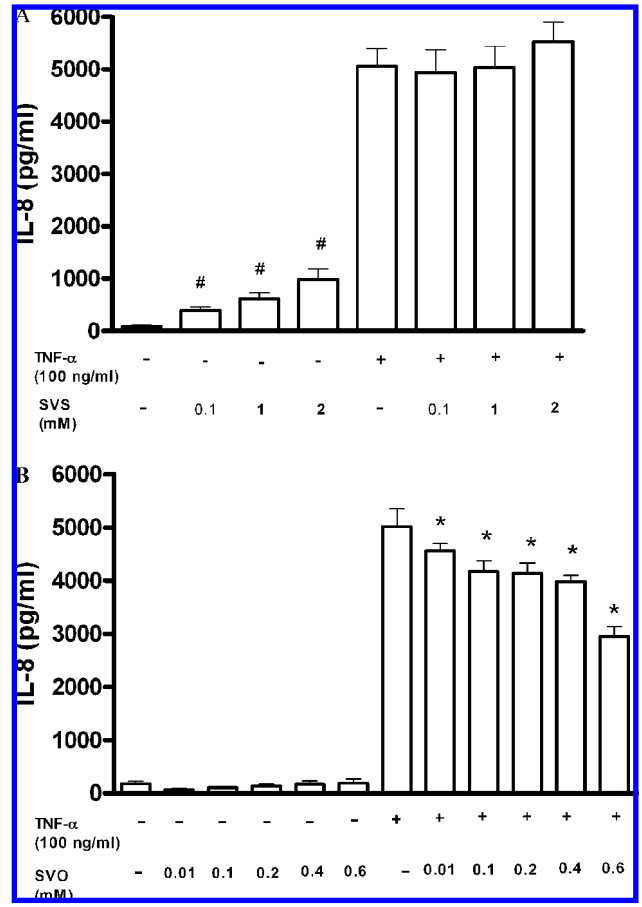

Figure 6. Effects of stevioside (**A**) and steviol (**B**) on the production of IL-8 in HT29 cells. Cells were treated for 8 h in the presence and absence of TNF-α as indicated. Data are expressed as the mean ± SEM of three independent experiments. (∗, #) Statistically significant difference in cytokine release ($p < 0.05$), as compared with TNF-α-treated and untreated group, respectively.

nmol/min. The cocktail of 8-Br-cAMP + forskolin + IBMX increased efflux to 225.2 ± 29.9, 278.2 ± 35.5, and 317.5 ± 75.3 nmol/min. Short-term exposure to steviol stimulated iodide efflux to 112.4 ± 15.3, 167.2 ± 20.9, and 301.5 ± 32.3 nmol/min at 3, 5, and 10 min. However, short-term exposure to stevioside caused only a modest increase in iodide efflux to 67.6 ± 4, 99.7 ± 6.3, and 164.4 ± 10.1 nmol/min.

DISCUSSION

Although stevioside and stevia extracts have been widely used as noncaloric sweeteners and as food supplements, there is less known about their mechanism of action in target tissues, including the intestine. Stevioside is converted to its major metabolite, steviol, by cecal and colonic, but not small intestinal, bacteria (*14, 16, 17*). Recent studies by Geuns et al. (*18, 19*) examined the metabolism of stevioside in healthy human volunteers. Whereas there was no detectable stevioside in plasma, urine, or feces, steviol was detected in the feces. Steviol glucuronide was detected in the plasma and urine, leading these authors to suggest that stevioside is fully metabolized to steviol in the colon and absorbed and transported to the liver for glucuronidation and excretion. It is therefore not surprising that the colonic cell line Caco-2 was shown to absorb steviol but not stevioside (*20*); however, no other biological function in the colon was assessed in that study. Because the colon appears to be the site of steviol generation, the present study examined the effects of stevioside and steviol in modulating two important functions of colonic epithelial cells: Cl^- secretion and pro-inflammatory cytokine production. We demonstrate that steviol has the more potent biological effect and increases Cl^- secretion (T84 cells) as well as attenuates TNF-α-mediated IL-8 release in the human colonic cell lines, T84, Caco-2, and HT29. In contrast, stevioside marginally increases Cl^- secretion, and at concentrations at which it does not affect cell viability, stevioside does not alter TNF-α function.

These results need to be examined in the context of other studies examining the effects of stevioside and steviol in vitro. Predictably, in hamster small intestine, steviol and not stevioside partially inhibited glucose absorption as reported earlier by Toskulkao et al. (*9*). This effect was observed at 1 mM, a concentration that altered intestinal morphology and depleted ATP. On the basis of the present study, steviol (0.2 and 0.8 mM) decreases cell viability to 80−90 and 7−34%, respectively, and it is conceivable that the changes in morphology and ATP reported in the hamster study could be a result of the loss of cell viability. In other animal models in vivo, orally fed stevioside 15 g/kg of body weight had little or no acute toxicity (*29*). On the basis of the evidence that stevioside is not acted upon by digestive enzymes (*14, 16, 18, 19*), it is most likely that the bulk of orally fed stevioside reaches the colon. In other in vitro studies, steviol (1 μM) and not stevioside (1 μM)

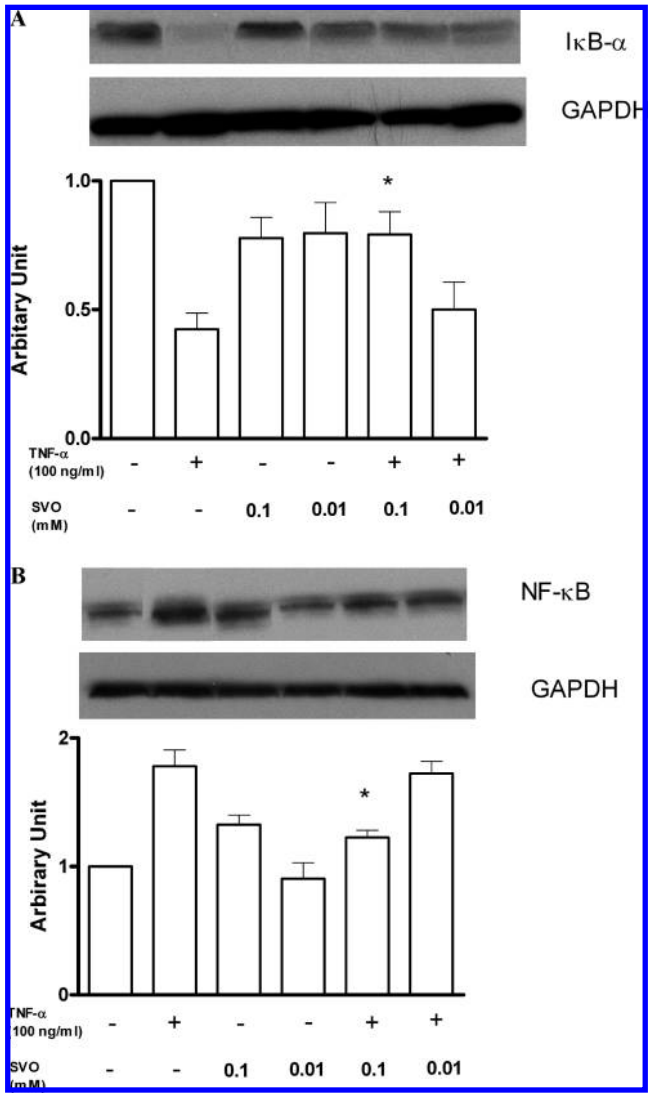

Figure 7. Effect of steviol on activation of IκB-α (**A**) and NF-κB (**B**) in T84 cells. Cells were treated for 45 min with TNF-α ± steviol at the indicated concentrations. (∗) Statistically significant difference in NF-κB and IκB-α protein expression ($p < 0.05$), as compared with TNF-α -treated group.

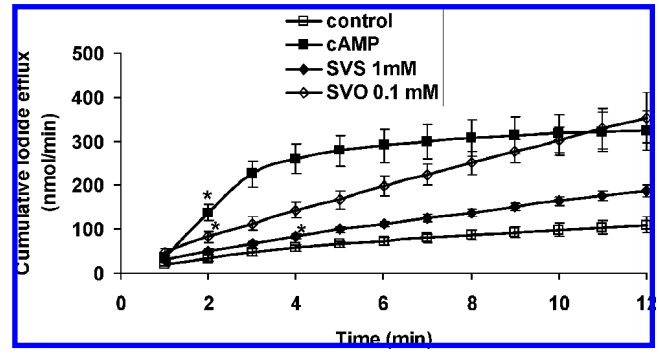

Figure 8. Effect of stevioside and steviol on iodide effluxes. T84 cells were treated with 0.2% DMSO (control), 8-Br-cAMP (100 μM) + forskolin (10 μM) + IBMX (100 μM), stevioside (1 mM), and steviol (0.1 mM). Data are expressed as the mean ± SEM of three independent experiments. (∗) Statistically significant difference in iodide efflux ($p < 0.05$), as compared with control group.

modulated organic anion transporters (hOAT1; SLC22A6 and hOAT3; SLC22A8) heterologously expressed in *Xenopus* oocytes (*30*). In contrast, in THP-1 human monocytes, stevioside and not steviol is known to have anti-inflammatory actions (*13*). Similarly, only stevioside affects glucose metabolism (*7*) in muscles from stevioside-fed rats or from naive rats, incubated in vitro with stevioside or steviol.

In most of these studies the effects of stevioside and steviol on cell viability were not assessed. The present studies in four intestinal cell lines (T84, HT29, Caco-2, and IEC-18 cells) underscore the importance of gauging the effects of stevioside and steviol on cell viability. Results demonstrate that 2 and 5 mM stevioside decreased cell viability to 76−82 and 33−68%, respectively. At much lower concentrations, 0.2 and 0.8 mM steviol also decreased cell viability to 80−90 and 7−34%, respectively. It is interesting that steviol, at 0.4 and 0.6 mM, and stevioside, at 2 and 5 mM, are less cytotoxic in HT29 and Caco-2 than in T84 cells (**Figure 2**). The temporary acceptable daily intake of 0−2 mg/kg of body weight of steviol equivalent (0−5 mg of stevioside) suggested by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) would result in approximately 300 mg of stevioside reaching the colon. Assuming that stevioside is not metabolized in any other part of the gastrointestinal tract, the final concentration in the colon could be as high as 0.187 mM, presuming a colonic volume of ≅2000 mL (*31*). However, the concentration of stevioside in the chyme will vary with the region of the gastrointestinal tract, and if the total secretions of the gastrointestinal tract are considered (7.5 L), the concentration could be as low as 0.05 mM. In addition, not all of the stevioside will reach the colon at the same time and is subject to bacterial metabolism. In either case, on the basis of the present studies and assuming that oral stevioside would be fully converted to steviol, concentrations of 0.05−0.2 mM stevioside would not be cytotoxic to colonocytes.

Because the colon is the only tissue where stevioside is metabolized to steviol and because little is known about the biological action of these compounds in the colon, their role in the regulation of Cl^- secretion and of proinflammatory cytokines in well-established colonic cell lines were explored. Colonic cell lines provide the advantage of a reductionist approach, but the findings must be interpreted with the caveat that the mammalian intestinal lumen is exposed to a constantly fluctuating environment of nutrients and toxins. In contrast to the small intestine, native colonic epithelial cells do not exhibit Na^+-dependent glucose transport. The normal healthy colon exhibits net fluid absorption, which represents a balance of net absorption of Na^+, Cl^-, and short-chain fatty acids and a secretion of K^+ and HCO_3^-. In addition, the colonocytes serve as the first line of defense between the microbe-rich lumen and the sterile subepithelial compartment. Colonic epithelial function is therefore tightly regulated, and the colonocytes often respond to luminal substances with an alteration in electrolyte transport and/or secretion of proinflammatory mediators. Thus, a disruption in the balance of colonic absorption and secretion favoring excessive water and electrolytes in the lumen could result in diarrhea. Calculations of the final concentrations of steviol in vivo will be complicated by factors including dilution, bacterial flora, and effects of the unstirred layer.

As shown in **Figure 8**, short-term (30 min) exposure to steviol and stevioside stimulated iodide efflux, with steviol (0.1 mM) having a much greater effect than stevioside (1 mM). The maximal efflux caused by steviol was similar to that evoked by the cAMP cocktail (8-Br-cAMP + forskolin + IBMX), although the time course was slower. The mechanisms underlying steviol action on Cl^- transport remain to be determined. It is unlikely

to involve NF-κB signaling as steviol alone did not alter this pathway (**Figure 7**).

Next, the effects of stevioside and steviol on immunomodulatory activities of colonic cells were examined. In the human monocytic THP-1 cells, stevioside, but not steviol, attenuates the immunomodulatory action of LPS by interfering with IKKβ → NF-κB signaling (*13*). TNF-α-mediated IL-8 release in T84, Caco-2, and HT29 cells was examined because the maximal response of these cells to LPS required 24 h. The time course and magnitude of IL-8 release in the three cell types is in keeping with earlier findings (*27, 28*) wherein its release from Caco-2 and HT29 cells was 2-fold greater than that from T84 cells.

At nontoxic concentrations, stevioside either alone or in the presence of TNF-α had no effect on IL-8 release. On the other hand, in the presence of TNF-α, steviol (0.01 and 0.1 mM) inhibited IL-8 release up to 21.1 and 35.4%. The percentage of inhibition is lower in Caco-2 and HT-29 cells and is perhaps due to the 2-fold higher baseline. At these concentrations steviol alone neither altered IL-8 release nor affected cell viability. These results are in marked contrast to THP-1 monocytes, where LPS stimulated TNF-α and IL-1β is decreased by stevioside, with steviol having no effect (*13*). However, in both monocytes and colonocytes, the attenuation of immunomodulator release by the stevia compounds is only partial (approximately 35%). The cell-specific differences between the effects of stevioside and steviol are puzzling and perhaps related to the expression of specific receptors.

TNF-α-mediated IL-8 release occurs by activation of the NF-κB signaling cascade. This well-characterized transcription factor is known to regulate the expression of a variety of genes including those that encode cytokines, chemokines, and cytokine receptors (*28*). Whereas steviol alone had no effect on NF-κB signaling, it attenuates TNF-α action by interfering with both IκB phosphorylation and NF-κB expression (**Figure 7**). In this aspect, steviol's actions are similar to the action of stevioside in THP-1 monocytes. Future studies will have to focus on determining whether steviol or stevioside can reduce inflammation in a model of experimental colitis.

In summary, we demonstrate for the first time that the natural metabolite of stevioside, steviol, has biological effects on colonic epithelial cells in terms of both electrolyte transport and immunomodulation. Our studies also underscore the cytotoxic effects of high concentrations of these drugs. Although the parent compound stevioside is known to affect biological function in a variety of cells, it is teleologically sound that the metabolite steviol, which is generated in the intestine, has its most potent effects in the gut. We suggest that long-term utilization of stevioside should take into consideration its role in the inflammatory and secretory response of colonocytes.

LITERATURE CITED

(1) Hanson, J. R.; De Oliveira, B. H. Stevioside and related sweet diterpenoid glycosides. *Nat. Prod. Rep.* **1993**, *10*, 301–309.

(2) Geuns, J. M. Stevioside. *Phytochemistry* **2003**, *64* (5), 913–921.

(3) Kinghorn, A. D.; Soejarto, D. D. Current status of stevioside as a sweetening agent for human use. In *Economic and Medical Plant Research*; Wagner, H., Hikino, H., Farmsworth, N. R., Eds.; Academic Press: London, U.K., 1985; Vol. 1, pp 1−52.

(4) Chan, P.; Tomlinson, B.; Chen, Y.; Liu, J.; Hsieh, M.; Cheng, J. A double blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *Br. J. Clin. Pharmacol.* **2000**, *50*, 215–220.

(5) Lee, C. N.; Wong, K; Liu, J.; Chen, Y.; Chen, J.; Chan, P. Inhibitory effect of stevioside on calcium influx to produce antihypertension. *Planta Med.* **2001**, *67*, 796–799.

(6) Jeppesen, P. B.; Gregersen, S.; Alsrupp, K. K.; Hermansen, K. Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects in vivo: studies in the diabetic goto-Kakizaki (GK) rats. *Phytomedicine* **2002**, *9*, 9–14.

(7) Lailerd, N.; Saengsirisuwan, V.; Sloniger, J. A.; Toskulkao, C. Effect of stevioside on glucose transport activity in insulin-sensitive and insulin-resistant rat skeletal muscle. *Metabolism* **2004**, *53*, 101–107.

(8) Jeppesen, P. B.; Kyrskog, S. E.; Agger, A.; Gregersen, Xiao, J.; Hermansen, K. Can stevioside in combination with a soy-based dietary supplement be a new useful treatment of type 2 diabetes? In vivo studies in the diabetic goto-Kakizaki Rat. *Rev. Diabet. Stud.* **2006**, *3* (4), 189–199.

(9) Toskulkao, C.; Sutheerawatananon, M.; Wanichanon, C.; Saitongdee, P.; Suttajit, M. Effect of stevioside and steviol on intestinal glucose absorption in hamsters. *J. Nutr. Sci. Vitaminol.* **1995**, *41*, 105–113.

(10) Jutabha, P.; Toskulkao, C.; Chatsudthipong, V. Effect of stevioside on PAH transport by isolated perfused rabbit renal proximal tubule. *Can. J. Physiol. Pharmacol.* **2000**, *78*, 737–744.

(11) Xi, Y.; Yamaguchi, T.; Sato, M.; Takeuchi, M. Antioxidant mechanism of *Stevia rebaudiana* extract and antioxidant activity of inorganic salts. *Nippon Kagaku Kaishi* **1998**, *45*, 317–322.

(12) Yasukawa, K.; Kitanaka, S.; Seo, S. Inhibitory effect of stevioside on tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. *Biol. Pharm. Bull.* **2002**, *25*, 1488–1490.

(13) Boonkaewwan, C.; Toskulkao, C.; Vongsakul, M. Anti-inflammatory and immunomodulatory activities of stevioside and its metabolite steviol on THP-1 Cells. *J. Agric. Food Chem.* **2006**, *54*, 785–789.

(14) Koyama, E.; Kitazawa, K.; Ohori, Y.; Izawa, Y.; Kakegawa, K.; Fuino, A.; Ui, M. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. *Food Chem. Toxicol.* **2003**, *41*, 359–374.

(15) Mosetting, E. Stevioside II. The structure of the aglycone. *J. Org. Chem.* **1995**, *20*, 884–899.

(16) Hutapea, A. M.; Toskulkao, C.; Buddhasukh, D.; Wilairat, P.; Glinsukon, T. Digestion of stevioside, a natural sweetener, by various digestive enzymes. *J. Clin. Biochem. Nutr.* **1997**, *23*, 177–186.

(17) Koyama, E.; Sakai, N.; Ohori, Y.; Kitazawa, K.; Izawa, O.; Kakekawa, K.; Fujino, A.; Ui, M. Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. *Food Chem. Toxicol.* **2003**, *41* (3), 875–883.

(18) Geuns, J. M.; Buyse, J.; Vankeirsbilck, A.; Temme, E. H.; Compernolle, F.; Toppet, S. Identification of steviol glucoronide in human urine. *J. Agric. Food Chem.* **2006**, *54* (7)), 2794–2798.

(19) Geuns, J. M.; Buyse, J.; Vankeirsbilck, A.; Temme, E. H. Metabolims of stevioside by healthy subjects. *Exp. Biol. Med.* **2007**, *232* (1), 164–173.

(20) Geuns, J. M.; Augustijins, P.; Mols, R.; Buyse, J. G.; Driessen, B. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. *Food Chem. Toxicol.* **2003**, *41* (11), 1599–1607.

(21) Adduci, J.; Buddhasukh, D.; Ternai, B. Improved isolation and purification of stevioside. *J. Sci. Soc. Thailand* **1987**, *12*, 179–183.

(22) Ogawa, T.; Nozaki, M.; Mitsui, M. Total synthesis of stevioside. *Tetrahedron* **1980**, *36*, 2641–2648.

(23) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65* (1−2), 55–63.

(24) Venglarik, C. J.; Bridges, R. J.; Frizzell, R. A. A simple assay for agonist-regulated Cl and K conductances in salt-secreting epithelial cells. *Am. J. Physiol.* **1990**, *259* (2 Part 1), C358–364.

(25) Chappe, V.; Hinkson, D. A.; Zhu, T.; Chang, X. B.; Riodan, J. R.; Hanarahan, J. W. Phosphorylation of protein kinase C sites in NBD1 and the R domain control CFTR channel activation by PKA. *J. Physiol.* **2003**, *548* (Part 1), 39–52.

(26) Ma, D.; Forsythe, P.; Bienenstock, J. Live *Lactobacillus reuteri* is essential for the inhibitory effect on tumor necrosis factor α-induced interleukin-8 expression. *Infect. Immun.* **2004**, *72* (9), 5308–5314.

(27) Porath, D.; Riegger, C.; Drewe, J.; Schwager, J. Epigallocatechin-3-gallate impairs chemokine production in human colon epithelial cell lines. *J. Pharmacol. Exp. Ther.* **2005**, *315*, 1172–1180.

(28) Elewaut, D.; DiDonato., J. A.; Kim, J. M.; Truong, F.; Eckmann, L.; Kagnoff., M. NF-κB is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. *J. Immunol.* **1999**, *163*, 1457–1466.

(29) Toskulkao, C.; Chaturat, L.; Temcharoen, P.; Glinsukon, T. Acute toxicity of stevioside, a natural sweetener, and its metabolite, steviol, in several animal species. *Drug Chem Toxicol.* **1997**, *20* (1−2), 31–44.

(30) Srimaroeng, C.; Chatsudthipong, V.; Aslamkhan, A. G.; Pritchard, J. B. Transport of the natural sweetener stevioside and its aglycone steviol by human organic anion transporter (hOAT1; SLC22A6) and hOAT3 (SLC22A8). *J. Pharmacol. Exp. Ther.* **2005**, *313* (2), 621–628.

(31) Iordanescu, G.; Pickhardt, P. J.; Choi, J. R.; Summers, R. M. Automated seed placement for colon segmentation in computed tomography colonography. *Acad. Radiol.* **2005**, *12* (2), 182–190.

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