The influence of 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) in caramel coloring III on gastrointestinal epithelial cells.

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ABSTRACT

Since the ingredients of soft drinks are generally hidden and protected by law, unknown substances may affect cell proliferation and the function of the gastrointestinal epithelial cells. Concerns have been expressed regarding the toxicity of caramel coloring used in soft drinks. The present study examined the potential toxicity of 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI), a byproduct of caramel coloring III, and its effects on three types of gastrointestinal cells (HGC-27, IEC-6, and Caco-2 cells). The inhibition of cell proliferation was stronger by Caramel III containing THI than by Caramel I or IV without THI. However, the inhibition of cell proliferation was not observed under THI exposure conditions, suggesting that THI does not act as a failure factor. On the other hand, since THI increased trans-epithelial electrical resistance (TEER), an indicator of epithelial physical barrier function, it may exert enhancing effects on tight junctions, a representative physical barrier. No cytotoxicity against gastrointestinal epithelial cells was observed, even under THI exposure conditions at concentrations that were markedly higher than those obtained from the ingestion of beverages. Conversely, TEER was elevated by the exposure to high THI concentrations, and was suggested to enhance epithelial cell barrier function. Since THI appears to act on absorption-regulating factors, further studies are needed, including methods for the application of THI.

KEY WORDS: 2-acetyl-4(5)-tetrahydroxybutylimidazole, caramel coloring III, gastrointestinal epithelial barrier function, P-glycoprotein, transepithelial electrical resistance

INTRODUCTION

Soft drinks (non-alcoholic beverages) are widely available to consumers because there are no age restrictions on their consumption and they may be purchased from retail stores and vending machines. The soft drink industry is constantly improving and innovating to create beverages for all to enjoy, and with the growing health consciousness of consumers in recent years, many diet-related and sugar-free products have been launched with increasing sales each year (1). Sales of soft drinks in Japan exceeded 4 trillion yen in 2018, but declined by 7% to 3,797.8 billion yen in 2020 due to the impact of the novel coronavirus (COVID-19). Nevertheless, the market is expected to grow in the future, indicating its size (2). In Japan, sales of cola are the highest followed by mineral water, tea, and coffee, accounting for more than 30% of all

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carbonated beverages. The global share of carbonated beverages is high, with cola accounting for the largest share of more than 50% (3).

Since the ingredients of soft drinks are generally hidden and protected by law, unknown substances may have an impact on cell proliferation and the function of gastrointestinal epithelial cells. We previously reported that relatively long-term exposure to Japanese Coca-Cola suppressed the proliferation of gastrointestinal epithelial cells (4). Cola is colored with caramel coloring III or IV, and we demonstrated that 4-methylimidazole (4-MeI), which is present in caramel coloring III or IV, may be the causative factor for growth inhibitory effects (5). However, since the concentration of 4-MeI needs to be markedly higher than the amount contained in Cola for it to exert growth inhibitory effects by itself, the mechanisms underlying this phenomenon have not yet been elucidated in detail.

On the other hand, 2-acetyl-4(5)-tetrahydroxy-butylimidazole (THI) is a reaction product formed in caramel coloring III, which is an ammonia caramel, similar to 4-MeI, which induces alveolar/bronchiolar adenoma and carcinoma in mice, as well as hyperinsulinemia, hypoglycemia, and mammary tumors in female rats (6-10), anorexigenic effects, DNA damage, and immunosuppressive effects, such as a decrease in lymphocytes, were detected with THI (11-14). On the other hand, the application of THI to the treatment of inflammatory bowel disease and prevention of splenic granuloma formation by utilizing its immunosuppressive effects has been proposed (15, 16). Since the findings described above showed the marked impact of THI on cells, it has been suggested to affect cell proliferation and epithelial cell barrier function in gastrointestinal cells.

While concerns have been expressed regarding the toxicity of 4-MeI and THI due to long-term and high-concentration exposure, their effects on gastrointestinal epithelial cells remain unknown. Therefore, the present study was performed to clarify the influence of THI on gastrointestinal epithelial cells and evaluate the safety of THI consumed from cola.

MATERIALS AND METHODS

Materials

Caramel coloring I, III, and IV were kindly gifted by Morita Food System Co., Ltd. (Mie, Japan). THI, 4-MeI, crystal violet, and 5(6)-carboxy fluorescein (5-CF), Rhodamine 123 (Rho123), and verapamil hydrochloride were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other reagents were of analytical grade or higher.

Cell culture

HGC-27, IEC-6, and Caco-2 cells were obtained from the Riken Cell Bank (Ibaraki, Japan) and kept in a humidified incubator at 37°C with 5% CO_2. HGC-27 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)-High glucose (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 250 ng/mL amphotericin B, and were used between passages 17-19. Caco-2 cells were maintained in DMEM-High glucose (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin, and were used between passages 19-21. IEC-6 cells were maintained in DMEM-High glucose (Wako, Osaka, Japan) supplemented with 5% fetal bovine serum, 4 mg/mL insulin, 100 U/mL penicillin, 100 mg/mL streptomycin, and 250 ng/mL amphotericin B, and were used between passages 9-11.

Proliferation assay

In the growth assay, each cell was seeded on 96-well culture plates (HGC-27 and IEC-6: 2.0×10^3 cells/well, Caco-2: 1.0×10^4 cells/well) and cultured for 24 hours to allow for adherence. Thereafter, the culture medium was replaced with fresh medium with/without the concentration of caramel coloring I, III, IV, THI, or 4-MeI indicated and then incubated for 1 or 96 h. Under 1-hour exposure conditions, the culture medium was exchanged with fresh medium after a 1-hour exposure to each substance and cells were then cultured for 96 hours.

To assess cell proliferation, relative cell numbers were measured using crystal violet staining for adherent cells. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, stained with 0.04% crystal violet aqueous solution for 15 min, and dissolved in 1% SDS. Cell viability was estimated by measuring absorbance using a microplate reader (ARVO MX 1420 MULTILABEL COUNTER, PerkinElmer Inc., MA, USA) at a wavelength of 560 nm.

Measurement of transepithelial electrical resistance (TEER)

Caco-2 cell monolayers (between passage numbers 20-22) were seeded on a Transwell insert (PET membrane, pore size of 0.4 mm) (Corning Inc., NY, USA) and cultured for 14–21 days. The integrity of cell monolayers was assessed by measuring TEER using a Millicell ERS testing device (Millipore corporation, MA, USA). After confirming the integrity of monolayers (TEER >400 Ωcm2), the culture medium was replaced with fresh DMEM. Ten minutes later, TEER was measured again and the culture medium was changed to DMEM including the indicated concentration of THI. TEER was measured at 0, 15, 30, 45, 60, and 120 minutes.

Membrane permeation experiments using transwell inserts

The transport of 5-CF and Rho123 across Caco-2 cell monolayers was examined using our previously described method (17). Caco-2 cell monolayers (between passage numbers 20-22) were seeded on transwell inserts (PET membrane, pore size of 0.4 mm) (Corning Inc., NY, USA). The integrity of cell monolayers was evaluated prior to the transport analysis by measuring TEER using a Millicell ERS testing device (Millipore Corporation, MA, USA). Monolayers with TEER >400 Ωcm^2 were used in membrane permeation experiments. In the apical to basal side direction, monolayers were washed twice using Hanks' balanced salt solution (HBSS buffer; pH 7.4; 10 mM HEPES) and then incubated in HBSS for 10 min. 5-CF (10 μM) or Rho123 (5 μM) was added to the apical side of the inserts (1.0 mL) and HBSS to the basal side (1.5 mL). One hundred microliters was withdrawn from the basal side at 0, 15, 30, 45, 60, 90, and 120 minutes, and was immediately replenished with an equal volume of pre-warmed HBSS. In the basal to apical side direction, monolayers were washed twice using HBSS buffer (pH 7.4; 10 mM HEPES) and then incubated in HBSS for 10 min. Rho123 (5 μM) was added to the basal side of a 12-well plate (1.5 mL) and HBSS to the apical side (1.0 mL). One hundred microliters was withdrawn from the apical side at 0, 15, 30, 45, 60, 90, and 120 min, and was immediately replenished with an equal volume of pre-warmed HBSS. The transepithelial transport of 5-CF and Rho123 across cell monolayers was assessed by SpectraMax iD5 (Molecular Devices, LLC, CA, USA).

Western blotting

The protein expression levels of P-glycoprotein (P-gp), Claudin-4, Claudin-7, and b-actin in cultured cells were evaluated by Western blotting. Samples were collected from cells after exposure to the indicated concentration of THI. Western blotting was performed using a monoclonal antibody for P-gp (P-glycoprotein Mouse mAb [C219], GeneTex), a polyclonal antibody for Claudin-4 (Claudin 4 Polyclonal Antibody, Bioss ANTIBODIES), a polyclonal antibody for Claudin-7 (Claudin 7 Polyclonal Antibody, Bioss ANTIBODIES), a polyclonal antibody for Occludin (Occludin Polyclonal Antibody, ABclonal), and a monoclonal antibody for b-actin (b-Actin Mouse mAb, Cell Signaling). P-gp, Claudin-4, Claudin-7, and Occludin expression levels in each cell were normalized by b-actin.

Statistical analysis

All results are expressed as the mean ± standard deviation (mean ± S.D.). Significant differences between groups were analyzed using Tukey's test and $P < 0.05$ was considered to be significant.

RESULTS

Color tone of each caramel coloring solution and comparison with Coca-Cola

The color tones of various caramel color solutions were investigated, shown in Figure 1. Caramel color I

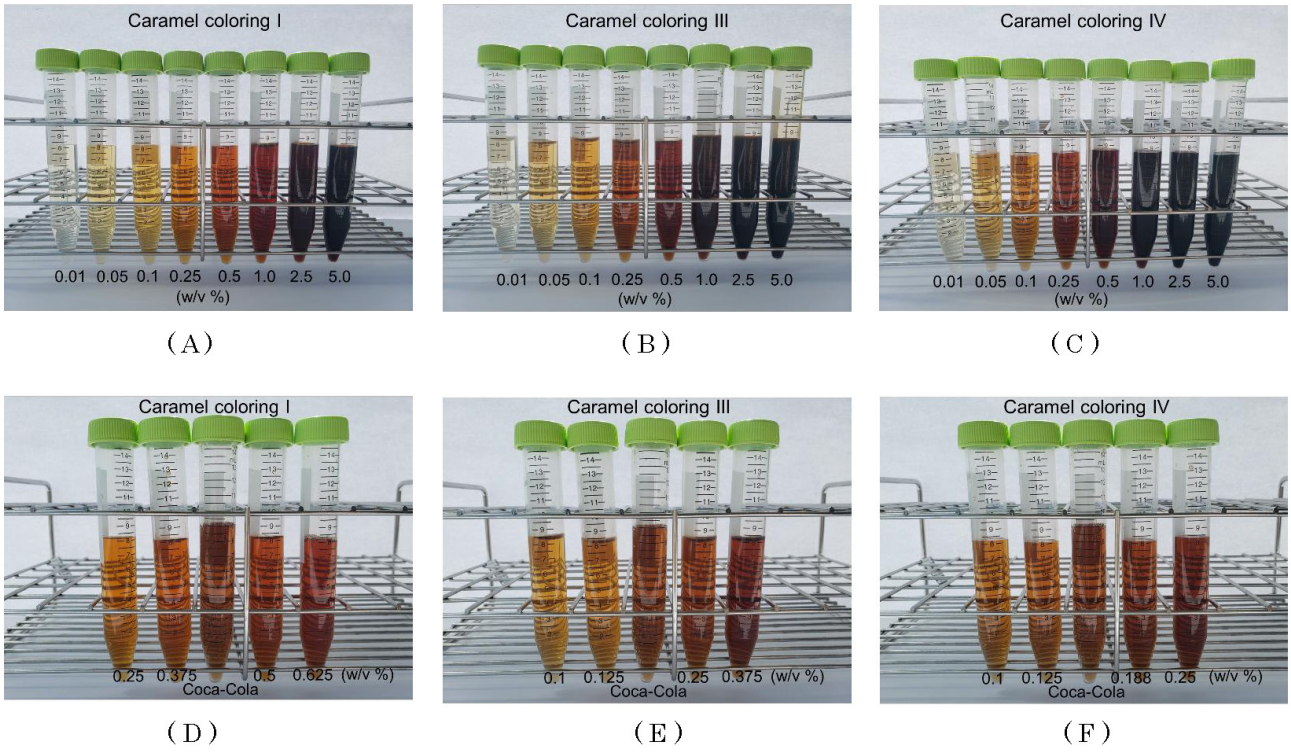


Figure 1 Color tone of each caramel coloring solution at several concentrations (caramel coloring I: A, caramel coloring III: B, caramel coloring IV: C), and comparisons with Coca-Cola (caramel coloring I: D, caramel coloring III: E, caramel coloring IV: F).

had a more reddish-brown color than caramel colors III and IV. On the other hand, caramel color IV was the darkest shown in Figures 1 A, B and C. The concentration of each caramel color was subsequently examined to achieve the same degree of coloration as Coca-Cola. Caramel color I, which has a strong reddish tint, was difficult to evaluate, but was shown to be approximately 0.4% (Figure 1 D). Caramel colors III and IV did not produce the same color as Coca-Cola, but were similar to that of Coca-Cola at approximately 0.2 and 0.18%, respectively (Figures 1 E and F).

The effects of caramel coloring on the proliferation of gastrointestinal epithelial cells

The concentrations of caramel coloring were set to 0.01 - 5 w/v% with a short exposure time of 1 hour and a long exposure time of 96 hours. The effects of caramel color on the growth rates of HGC-27, IEC-6, and Caco-2 cells were then examined.

Although the proliferation of Caco-2 cells exposed to caramel colors for 1 hour remained unchanged (Figure 2 E), the inhibitory effects of caramel colors on cell proliferation occurred in a concentration-dependent manner under other conditions as shown in Figures 2 A, B, C, D, and F). Furthermore, inhibitory effects on cell proliferation were the weakest for caramel coloring I and the strongest for caramel color III.

Effects of THI on the proliferation of gastrointestinal epithelial cells

As shown in Figure 2, the effects of THI (0.59 - 10 μg/mL) on cell proliferation were examined using three types of gastrointestinal epithelial cells. Similar to caramel coloring, the exposure times for THI were set to a short exposure time of 1 hour and a long exposure time of 96 hours.

Under all experimental conditions, THI exposure did not significantly inhibit cell proliferation in a time-

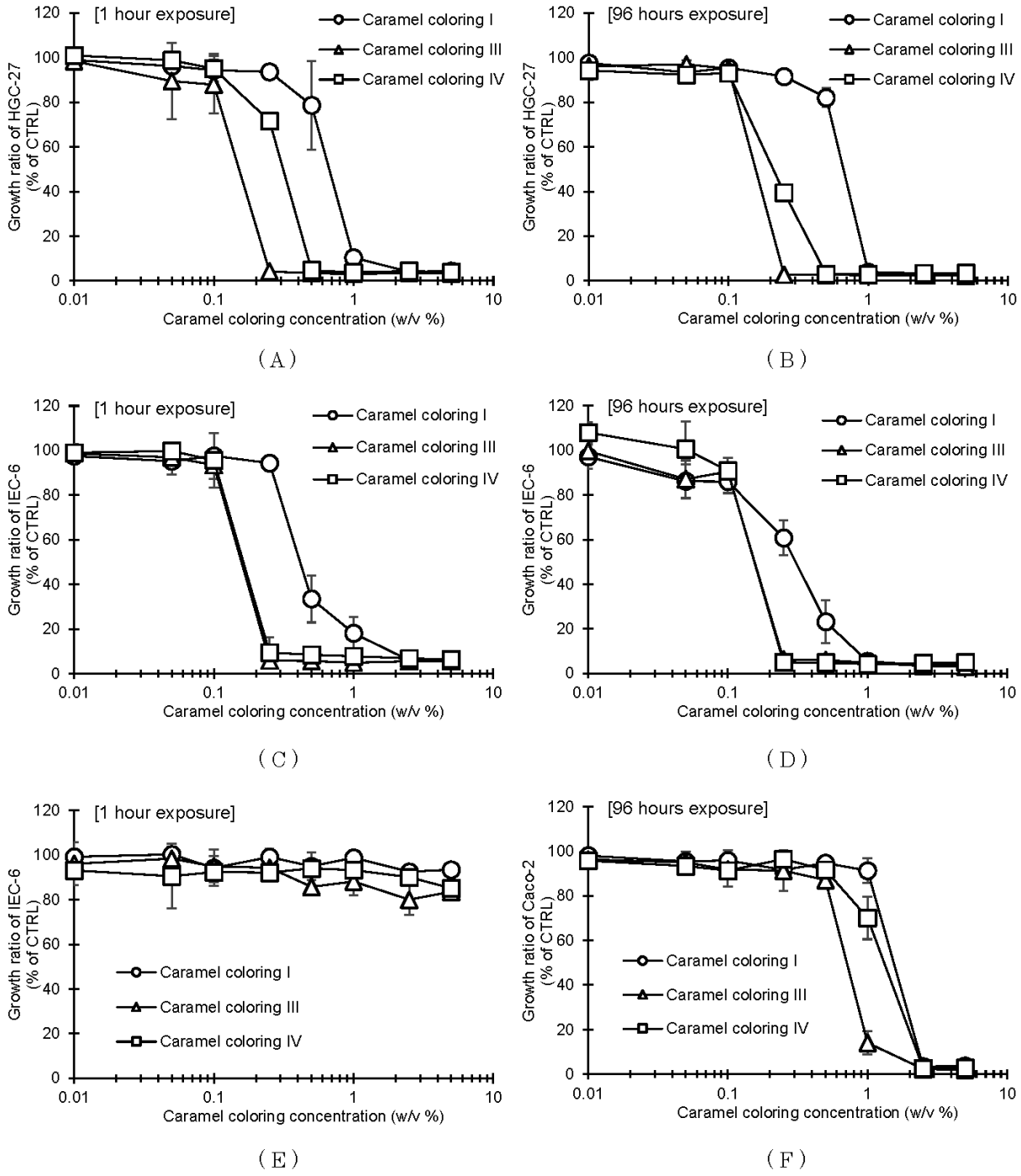


Figure 2 Growth rates of HGC-27 (A and B), IEC-C (C and D), and Caco-2 (E and F) cells under caramel coloring I, III, and IV exposure conditions (0.01 to 5 w/v%). Exposure times to caramel coloring were (A, C, and E) 1 hour and (B, D, and F) 96 hours. Data represent means ± S.D. (n = 6 for each condition).

or concentration-dependent manner (Figure 3). A combination of 4-MeI (0.09 - 200 μg/mL) and THI (10 μg/mL) was examined, and the significant effects of the THI co-exposure on the time- and concentration-dependent inhibition of cell proliferation by 4-MeI were not observed (Figure 4).

The influence of THI on the epithelial barrier function of Caco-2 cells

The effects of THI exposure on gastrointestinal epithelial barrier function were investigated. Although the TEER of Caco-2 cells did not change with the exposure to THI at 1 μg/mL, TEER became larger at 10 μg/mL and significantly increased over that under CTRL conditions from 48 hours after exposure (Figure 5A). Furthermore, membrane permeation experiments using Caco-2 cells, which were exposed to THI for 96 hours, showed that the membrane-permeated amount of 5CF, a paracellular marker, under 1 μg/mL THI exposure conditions was similar to that under CTRL conditions, whereas that under 10 μg/mL THI exposure conditions was significantly lower than that under CTRL conditions (Figure 5B). In apical (A) to basal (B) and B to A direction, the membrane permeability of Rho123 was increased and decreased by Verapamil which is a typical P-gp inhibitor, respectively, so it was confirmed that P-gp was working in Caco-2 cell monolayers. But the membrane permeability of Rho123, a typical P-gp substrate, showed no significant changes in absorption (A to B) or excretion (B to A) directions under similar THI exposure conditions (Figures 5 C and D).

On the other hand, the protein expression levels of membrane proteins involved in epithelial barrier function, such as P-gp, Claudin-4, Claudin-7, and Occludin, after exposure to THI for 48 and 96 hours were not significantly altered (Figures 6 A and B).

DISCUSSION

There are four types of caramel colors namely I, II, III, and IV. Caramel color III is used for cola, and the concentration of caramel color in beverages ranges from 0.01 to 6 w/v%. Initially the color tones of caramel coloring solutions for caramel I, III, and IV were examined. Caramel color III and IV solutions of approximately 0.2% showed a similar color tone to that of Coca-Cola (Figure 1).

Soft drinks pass through the esophagus, stomach, small intestine, and large intestine. To clarify the effects of caramel coloring ingested as an additive in soft drinks on gastrointestinal epithelial cells, HGC-27 (a human stomach carcinoma cell line), IEC-6 (a rat normal small intestine epithelial cell line), and Caco-2 (a human colon adenocarcinoma cell line) cells were selected as gastrointestinal epithelial cells.

The production methods for each caramel color are as follows: caramel color I is produced by superheating sugar, caramel color III by heating sugar with an ammonia compound, and caramel color IV by heating sugar with sulfurous acid and an ammonium compound. As described above, caramel color III contains 4-MeI and THI as byproducts, while caramel color IV contains 4-MeI as a byproduct. As shown in Figure 2 and Table 1, the IC_{50} of each caramel color on cell proliferation was I>IV>III for all three types of cells, indicating that caramel coloring III exerted the strongest inhibitory effects on cell proliferation and is highly toxic. Since the inhibition of cell proliferation

Table 1 IC50 of Caramel coloring I, III, and IV on the proliferation of HGC-27, IEC-6, and Caco-2 cells.

	CARAMEL COLORING I		CARAMEL COLORING III		CARAMEL COLORING IV	
	1 h Exposure	96 h Exposure	1 h Exposure	96 h Exposure	1 h Exposure	96 h Exposure
HGC-27	0.67 %	0.66 %	0.15 %	0.16 %	0.31 %	0.21 %
IEC-6	0.41 %	0.30 %	0.16 %	0.16 %	0.16 %	0.16 %
Caco-2	> 5.0 %	1.54 %	> 5.0 %	0.71 %	> 5.0 %	1.31 %

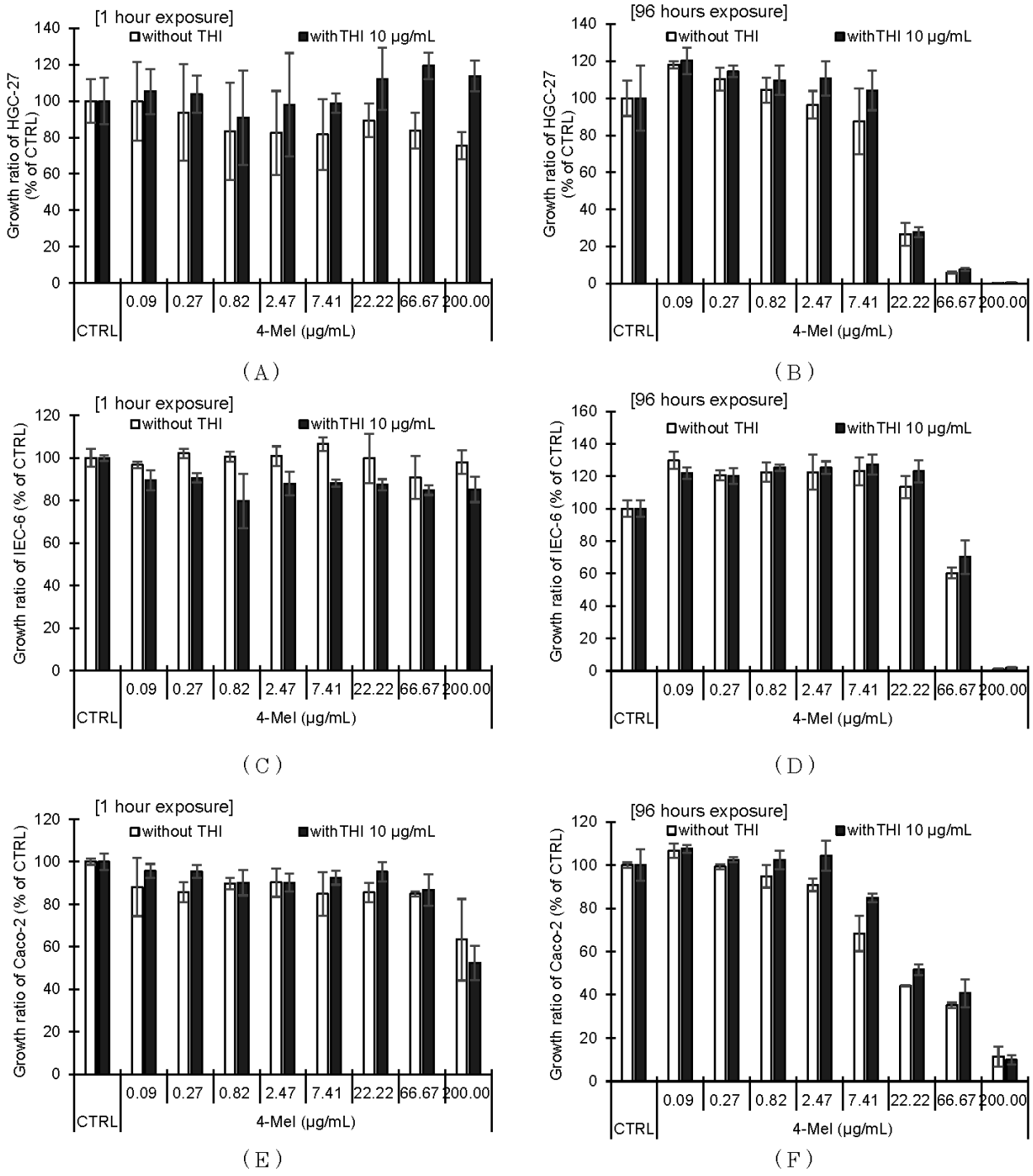


Figure 4 Growth rates of HGC-27 (A and B), IEC-C (C and D), and Caco-2 (E and F) cells under 4-MeI (0.09 – 200 μg/mL) and/or THI (10 μg/mL) exposure conditions. Exposure times to 4-MeI and/or THI were (A, C, and E) 1 hour and (B, D, and F) 96 hours. Data represent means ± S.D. (n = 6 for each condition).

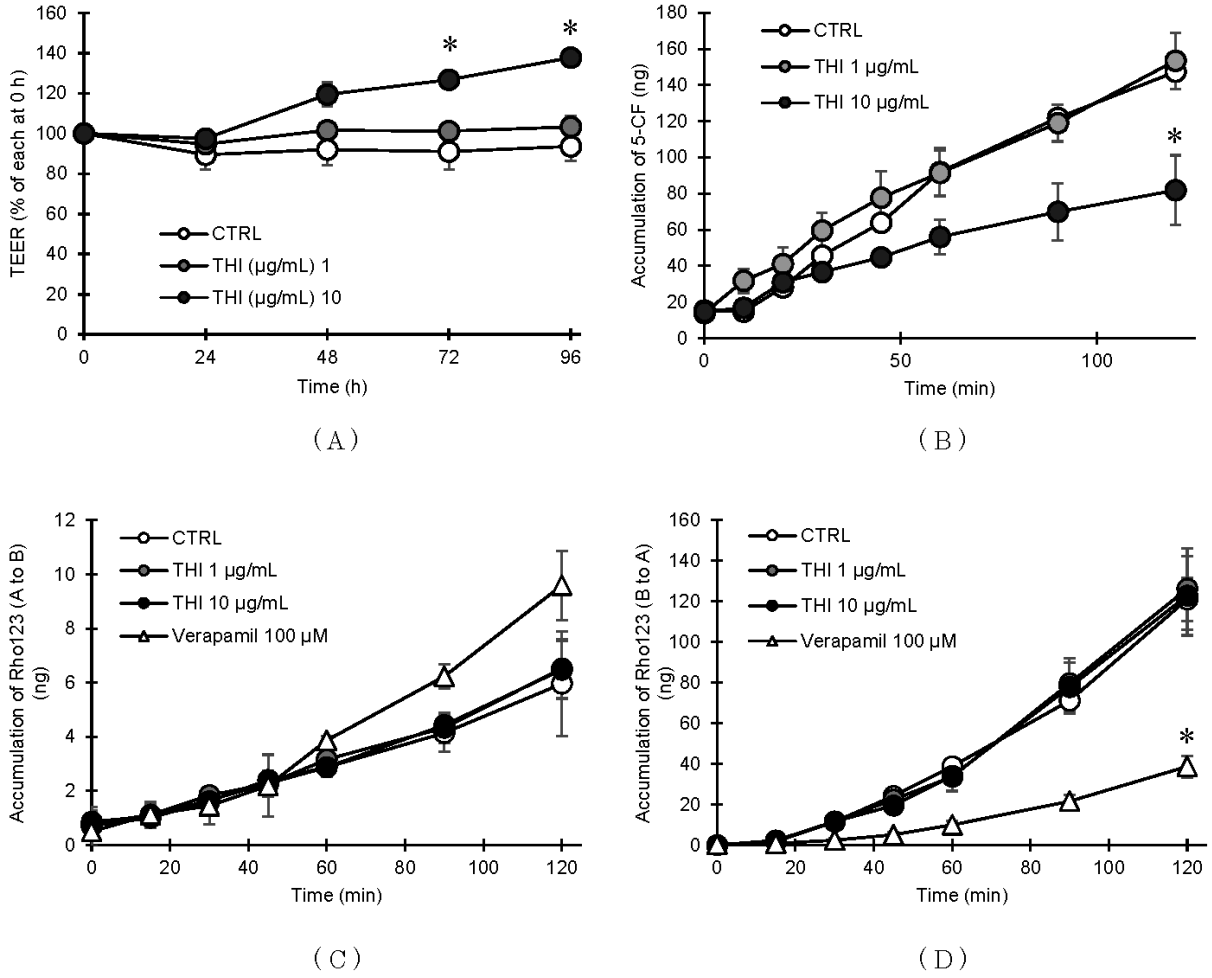
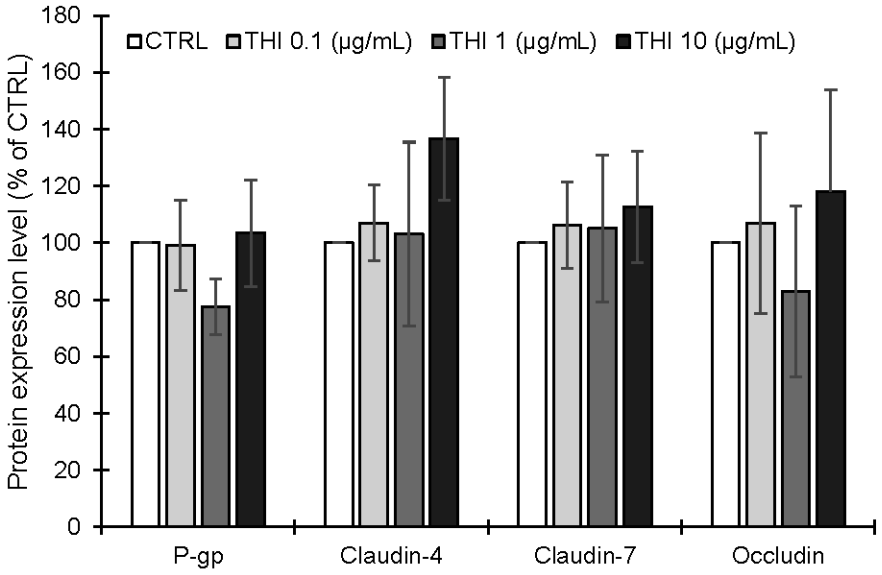


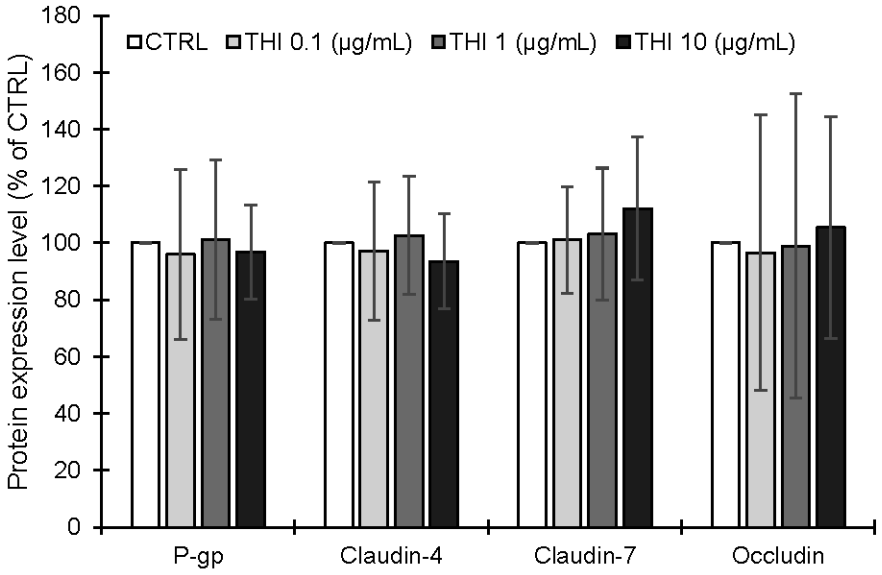
Figure 5 Effects of THI (1 and 10 μg/mL) on TEER (A), and the membrane permeation of 5-CF (B) and Rho123 (C and D) in Caco-2 cells. The direction of the membrane permeation of Rho123 is the apical to basal side (C) and the basal to apical side (D). Results represent mean and S.D. (n = 4-6). *P<0.05 vs. CTRL conditions.

by caramel colors III and IV, which contain 4-MeI and THI as byproducts, was stronger than that by caramel color I, 4-MeI and THI may act as inhibitory factors for proliferation. Moreover, it has previously been reported that the inhibitory effects of 4-MeI on cell proliferation at concentrations that were markedly higher than the clinical exposure concentration (5). However, the present results revealed the inhibitory effects of caramel color III, which contains THI. Therefore, THI also appears to be an important failure factor. To clarify the toxicity of THI against gastrointestinal epithelial cells, the effects of an exposure to THI on gastrointestinal cells were investigated (Figure 3). Since the content of THI in caramel color III is 40 μg/g or less in Japan (18), the maximum concentration of THI in a 0.2% solution of caramel color III is 0.08 μg/mL. On the other hand, the maximum concentration of 4-MeI, which may also be a failure factor, is 300 μg/g or less in caramel coloring III (18); therefore, the maximum concentration of 4-MeI in a 0.2% solution of caramel color III is 0.6 μg/mL.

Since 4-MeI of caramel color IV is specified as 1,000 μg/g or less (18), the maximum concentration of 4-MeI



(A)



(B)

Figure 6 P-glycoprotein (P-gp), Claudin-4, Claudin-7, and Occludin protein expression levels in Caco-2 cells after 48 (A) and 96 hours (B) of exposure to THI (0.1, 1 and 10 µg/mL). Data represent means ± S.E. (n = 3 for each condition).

in a 0.2% solution of caramel color IV is 2.0 μg/mL. Based on these concentrations of caramel colors, the present study was conducted with caramel colors, THI, and 4-MeI from expected exposure concentrations *in vivo* during the consumption of a soft drink containing caramel coloring, such as Coca-Cola, to a markedly higher concentration range.

When the three types of gastrointestinal epithelial cells were exposed to THI at a maximum concentration of 10 μg/mL, a significant effect on cell proliferation was not observed (Figure 3). This result suggested that THI alone did not affect the proliferation of gastrointestinal cells. Furthermore, since THI did not enhance the inhibitory effects of 4-MeI on cell proliferation, it did not appear to exert additive or synergistic effects on the inhibitory effects of 4-MeI on cell proliferation (Figure 4). These results indicate that the strong inhibitory effects of caramel color III on cell proliferation shown in Figure 2 and Table 1 were not due to THI, but to other factors. Further studies are needed to establish whether THI interacts with other substances in caramel coloring III in order to exert its inhibitory effects on cell proliferation.

Although THI alone did not exert inhibitory effects on cell proliferation, other factors may also have been affected by THI. When THI is used to treat and prevent a disease, as described in the Introduction section (15, 16), it may be used in combination with other drugs. Therefore, the present study investigated the effects of THI exposure on intestinal epithelial barrier function, which regulates the membrane permeation of drugs and nutrients in the intestines.

By focusing on tight junctions (TJ) as a physical barrier function of epithelial cells on the gastrointestinal tract, the effects of THI on TEER were examined, TJ intensity, and the permeation of 5-CF, a paracellular marker. Under 10 μg/mL THI exposure conditions, TEER increased from 48 hours after exposure, and significantly increased at 72 and 96 hours (Figure 5 A). Correspondingly, a significant decrease in the membrane permeation of 5-CF was detected (Figure 5 B). However, the protein expression levels of Claudin family (Claudin-4 and Claudin-7) and Occludin, which are constituent components of TJ, did not change even under 10 μg/mL THI exposure conditions (Figure 6). Although the effects of THI exposure on TJ, one of the physical barrier functions of epithelial cells, was suggested, a correlation was not observed between the enhancement of TJ function and changes in the expression levels of its constitutive factors. However, since the regulation of TJ involves the expression levels and ratios of the molecular species of Claudin family members (19-21), the regulation of TJ cannot be evaluated based solely on the expression levels of specific species of the Claudin family. The mechanisms by which THI increases TEER may be elucidated in further studies that include measurements of the expression levels and localization of other Claudin families. On the other hand, the effects of THI on P-gp, which is the most important excretion transporters and one of the biological barriers of epithelial cells, were examined. No significant changes were detected in the membrane permeation of Rho123, a typical P-gp substrate whose membrane permeation is less sensitive to changes in TJ, even after exposure to 10 μg/mL for 96 hours (Fig. 5C, D), and P-gp protein expression levels remained unchanged (Fig. 6). Therefore, THI did not appear to exert any specific effects on P-gp at least under the limited condition of this study.

CONCLUSIONS

The present study focused on and investigated THI, which is a byproduct of caramel color III, as a toxic factor against gastrointestinal cells. However, cytotoxicity against gastrointestinal epithelial cells were not observed in the *in vitro* study, even under THI exposure conditions at concentrations that were markedly higher than those ingested from the consumption of beverages. Conversely, TEER was elevated by an exposure to high concentrations of THI and appeared to enhance epithelial cell barrier function. Since TJ regulate membrane permeation via a paracellular route, they are also an absorption-regulating factor. In other words, THI may act on absorption-regulating factors, and, thus, further studies are needed that include methods for the application of THI.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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