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Effects of A GLP-1 Receptor Agonist on Gastrointestinal Epithelial Cells

Yusuke Takizawa[1*], Junya Oguri[1], Masaya Uno[1], Ami Onsui[1], Atsushi Ishimura[2], Takuro Kurita[1], Takanori Nakajima[1]

[1]Division of Clinical Pharmaceutics, Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, 10281 Komuro, Inamachi, Kitaadachi-gun, Saitama 362-0806, Japan
[2]Division of Clinical Pharmacy, Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, 10281 Komuro, Inamachi, Kitaadachi-gun, Saitama 362-0806, Japan

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***Corresponding author:** Yusuke Takizawa, Ph.D.
Division of Clinical Pharmaceutics, Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, 10281 Komuro, Inamachi, Kitaadachi-gun, Saitama 362-0806, Japan

Abstract **Original Research Article**

The number of people with diabetes worldwide has increased to 537 million, with a 3.6-fold increase occurring in the last 20 years. Various medications are used in the treatment of diabetes, and glucagon-like peptide-1 (GLP-1) receptor agonists have been recommended in major overseas guidelines. Although the main route of administration of GLP-1 receptor agonists is subcutaneous, orally administered GLP-1 receptor agonists were approved in 2019. Therefore, gastrointestinal epithelial cells are exposed to GLP-1 receptor agonists with poor bioavailability; however, it currently remains unclear whether this luminal approach damages these cells. The present study investigated the effects of GLP-1 receptor agonists on gastrointestinal epithelial cells. The gastrointestinal epithelial cell lines HGC-27, IEC-6, and Caco-2 were treated with Liraglutide, a GLP-1 receptor agonist. The protein expression of the GLP-1 receptor was confirmed in these cell lines, and Liraglutide did not significantly affect their proliferation. Although no inhibitory effects on oxidative stress were noted, Liraglutide appeared to alter the expression of drug absorption-regulating factors. In conclusion, since no significant changes were detected in the cell lines examined, even at higher concentrations of Liraglutide, there were no significant effects at the cellular level. However, since the present results were contradictory to previous findings, further studies using different conditions and cells are warranted.

Keywords: GLP-1 receptor agonist, gastrointestinal epithelial cell, oral administration, Liraglutide, Caco-2.

INTRODUCTION

The number of people with diabetes worldwide has increased to 537 million, with a 3.6-fold increase occurring in the last 20 years [1]. The prevalence of diabetes has reached 10.5%, and 1 out of 10 adults are currently living with diabetes; however, approximately half (44.7%) are undiagnosed. There are two types of diabetes, type 1 and type 2 diabetes, and more than 90% of diabetics have the latter. Type 2 diabetes is caused by genetic factors that predispose individuals to low insulin secretion and insulin resistance, combined with lifestyle factors, such as overeating and a lack of exercise. Common reasons for the increased prevalence of type 2 diabetes include urbanization, aging, a lack of exercise and physical inactivity, and an increase in the number of overweight and obese individuals.

Drug therapy for diabetes starts with insulin injections, followed by alpha-glucosidase inhibitors, thiazolidinediones, fast-acting insulin secretagogues, including glinides, and sulfonylurea drugs. In recent years, a series of incretin-related drugs classified into two categories, glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 inhibitors, have been launched in the market and has expanded therapeutic options. GLP-1 receptor agonists are recommended for the treatment of diabetes in major overseas guidelines. GLP-1, an incretin hormone along with gastric inhibitory peptide (GIP), is synthesized by L-cells in the distal small intestine and is secreted in response to the intake of food [2]. GLP1 analogues have potential as anti-diabetic drugs that exert favorable effects on bone metabolism in clinical practice [3]. Liraglutide is a clinical agent with an acylated GLP-1 analog that may improve glycemic control in patients with diabetes.

Although the main route of administration of GLP-1 receptor agonists is subcutaneous, orally administered GLP-1 receptor agonists (Ozempic, Semaglutide, Novo Nordisk Pharma Ltd.) were

approved in the United States in 2019 and in Japan in 2020. Since GLP-1 receptor agonists have been shown to not only improve blood glucose levels, but also reduce body weight [4], they are used as diet medication in the United States and may be taken by non-diabetic patients. Therefore, the use of orally administered formulations of GLP-1 receptor agonists will continue to increase in the future.

GLP-1 receptor agonists not only exert therapeutic effects on diabetes, they have also been reported to exhibit anti-inflammatory activity in humans and rodent pathological models [5, 6, 7], improve lipid metabolism and promote weight loss in patients with non-alcoholic fatty liver disease [8, 9], and are therapeutically beneficial for asthma [10]. However, the side effects of Liraglutide, a GLP-1 receptor agonist, include drug-induced hepatotoxicity [11], increased oxidative stress [12], and acute kidney injury [13]. Therefore, the effects of GLP-1 receptor agonists on cells are controversial.

The findings described above were based on the effects of a plasma membrane approach after subcutaneous administration into the blood. Gastrointestinal epithelial cells are exposed to orally administered GLP-1 receptor agonists via a luminal approach and it currently remains unclear whether this route induces cellular damage. Since the bioavailability of orally administered formulations of GLP-1 receptor agonists is less than 1% [14], the majority of an ingested GLP-1 receptor agonist will remain in the gastrointestinal tract and continue to be exposed to gastrointestinal epithelial cells. GLP-1 receptor agonists were previously reported to be cytotoxic at high concentrations [12].

Limited information is currently available on the expression of the GPL-1 receptor in gastrointestinal epithelial cells; therefore, further studies are needed to clarify the effects of GLP-1 receptor agonists on gastrointestinal epithelial cells and confirm their safety. The present study investigated the effects of GLP-1 receptor agonists on gastrointestinal epithelial cells.

MATERIALS AND METHODS

Materials

Victoza Subcutaneous Injection was purchased from Novo Nordisk Pharma Ltd. (Tokyo, Japan). Crystal violet and hydrogen peroxide (H_2O_2) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were of analytical grade or higher.

Cell culture

The MCF-7, HGC-27, IEC-6, and Caco-2 cell lines were obtained from the Riken Cell Bank (Ibaraki, Japan) and kept in a humidified incubator at 37°C with 5% CO_2. MCF-7 and 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 μg/ml streptomycin, and 250 ng/ml amphotericin B, and were used between passages 21 - 26 and 25 - 29, respectively. IEC-6 cells were maintained in DMEM-High glucose (Wako, Osaka, Japan) supplemented with 5% fetal bovine serum, 4 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B, and were used between passages 16 - 21. Caco-2 cells were maintained in DMEM-High glucose (Fujifilm Wako, Osaka, Japan) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 U/mL of penicillin, 100 μg/ml of streptomycin, and 250 ng/mL of amphotericin B, and were used between passages 12 - 18.

Proliferation assay

In the growth assay, each cell was seeded on a 96-well culture plate (MCF-7, HGC-27, and IEC-6: 2.0×10^3 cells/well, Caco-2: 1.0×10^4 cells/well) and cultured for 24 h to adhere to the culture plate. Thereafter, the culture medium was replaced with fresh medium with/without each concentration of Liraglutide and incubated for 96 h.

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 min, stained with 0.04% crystal violet aqueous solution for 30 min, and dissolved in 1% SDS solution. Cell viability was estimated by measuring absorbance on a microplate reader (ARVO MX 1420 MULTILABEL COUNTER, PerkinElmer Inc., MA, USA) at a wavelength of 560 nm.

Cell viability assay

In the growth assay, each cell was seeded on a 96-well culture plate (MCF-7, HGC-27, and IEC-6: 2.0×10^3 cells/well, Caco-2: 1.0×10^4 cells/well) and cultured to confluency (approximately 96 h). Cells were then treated with each concentration of Liraglutide and incubated for 4 h. Cultured medium was removed and Liraglutide was added with/without H_2O_2. Cells were then incubated for 24 h.

To assess cell viability, relative cell numbers were measured using crystal violet staining for adherent cells. Cells were fixed with 4% paraformaldehyde in PBS for 10 min, stained with 0.04% crystal violet aqueous solution for 30 min, and dissolved in 1% SDS solution. Cell viability was estimated by measuring absorbance on a microplate reader (ARVO MX 1420 MULTILABEL COUNTER, PerkinElmer Inc., MA, USA) at a wavelength of 560 nm.

Western blotting

The protein expression levels of GLP-1R, caspase-9, HIF-1α, claudin-4, mucin-1 (MUC1), P-gp, and β-actin in cultured cells were evaluated by Western

blotting. Western blotting using monoclonal antibodies for GLP-1R (GLP-1R Rabbit mAb, ABclonal), caspase-9 (Caspase-9 Rabbit mAb, ABclonal), HIF-1α (HIF-1α Rabbit mAb, ABclonal), claudin-4 (Claudin-4 Mouse mAb, Invitrogen), MUC1 (MUC1 Rabbit pAb, ABclonal), P-gp (P-glycoprotein Mouse mAb [C219], GeneTex), and β-actin (β-Actin Mouse mAb, Cell Signaling) was performed. Each protein expression level was normalized by β-actin.

STATISTICAL ANALYSIS

All results are expressed as the mean ± standard deviation (S.D.). The significance of differences between groups was analyzed using the Student's *t*-test. P <0.05 was considered to be significant.

RESULTS AND DISCUSSION

Protein Expression of the GLP-1 Receptor in Gastrointestinal Cells

The target of GLP-1 receptor agonists is the GLP-1 receptor; however, limited information is currently available on the expression of the GLP-1 receptor in gastrointestinal epithelial cells. GLP-1 receptor agonists, such as Liraglutide, are not taken up intracellularly. If the GLP-1 receptor is expressed in gastrointestinal epithelial cells, its binding site may face the extracellular side; therefore, the expression of the GLP-1 receptor needs to be confirmed in order to examine the effects of Liraglutide on these cells.

Although the mRNA expression of the GLP-1 receptor has been demonstrated in the ileum and colon [15-17], its protein expression level in human gastrointestinal cells remains unknown. Therefore, we herein investigated whether the GLP-1 receptor was expressed in the gastrointestinal epithelial cell lines HGC-27, IEC-6, and Caco-2. MCF-7 cells, in which the expression of the GLP-1 receptor was previously demonstrated [18], were used as a positive control in the present study and GLP-1 receptor expression was confirmed. The expression of the GLP-1 receptor was detected in HGC-27, IEC-6, and Caco-2 cells (Fig. 1), and its levels were higher in these cell lines than in MCF-7 cells.

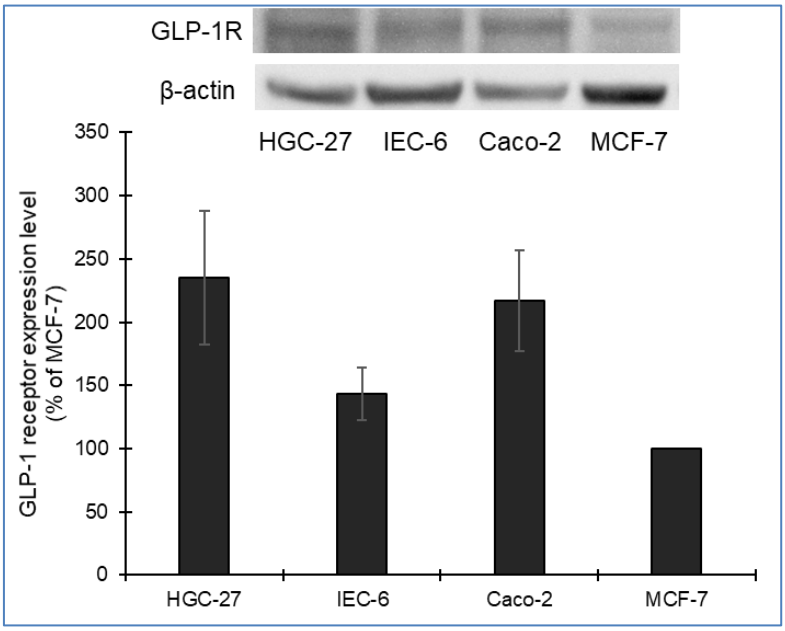

Fig-1: Protein expression levels of the GLP-1 receptor (GLP-1R) in gastrointestinal cells. Data represent means and S.D. (n = 4 for each cell line)

The protein expression level of the GLP-1 receptor was confirmed in the three gastrointestinal epithelial cell lines examined in the present study. GLP-1 receptor expression was previously shown to be induced by GLP-1 receptor agonists [19]. On the other hand, GLP-1 receptor expression was also induced at the protein level in an inflamed rat colon [20]. Since GLP-1 receptor expression levels appear to vary under different conditions, further studies on other cells and conditions, including digestive diseases, are needed.

Effects of Liraglutide on the Proliferation of Gastrointestinal Cells

The bioavailability of orally administered formulations of GLP-1 receptor agonists is less than 1% [14]; therefore, the majority of an ingested GLP-1 receptor agonist will remain in the gastrointestinal tract and continue to be exposed to gastrointestinal epithelial cells. Although GLP-1 receptor agonists are considered to be safe for oral administration to patients [21], their effects at the cellular level remain unknown. In the present study, Liraglutide was used as a GLP-1 receptor

agonist, and its effects on the proliferation of gastrointestinal epithelial cells were examined.

The results obtained showed no significant changes in the three gastrointestinal epithelial cell lines treated with Liraglutide (Fig. 2A, 2B, 2C). However, the growth of IEC-6 cells was slightly inhibited by Liraglutide in a concentration-dependent manner. On the other hand, Liraglutide did not affect the proliferation of MCF-7 cells, which also express the GLP-1 receptor, at least until a concentration of 20,000 nM (Fig. 2D).

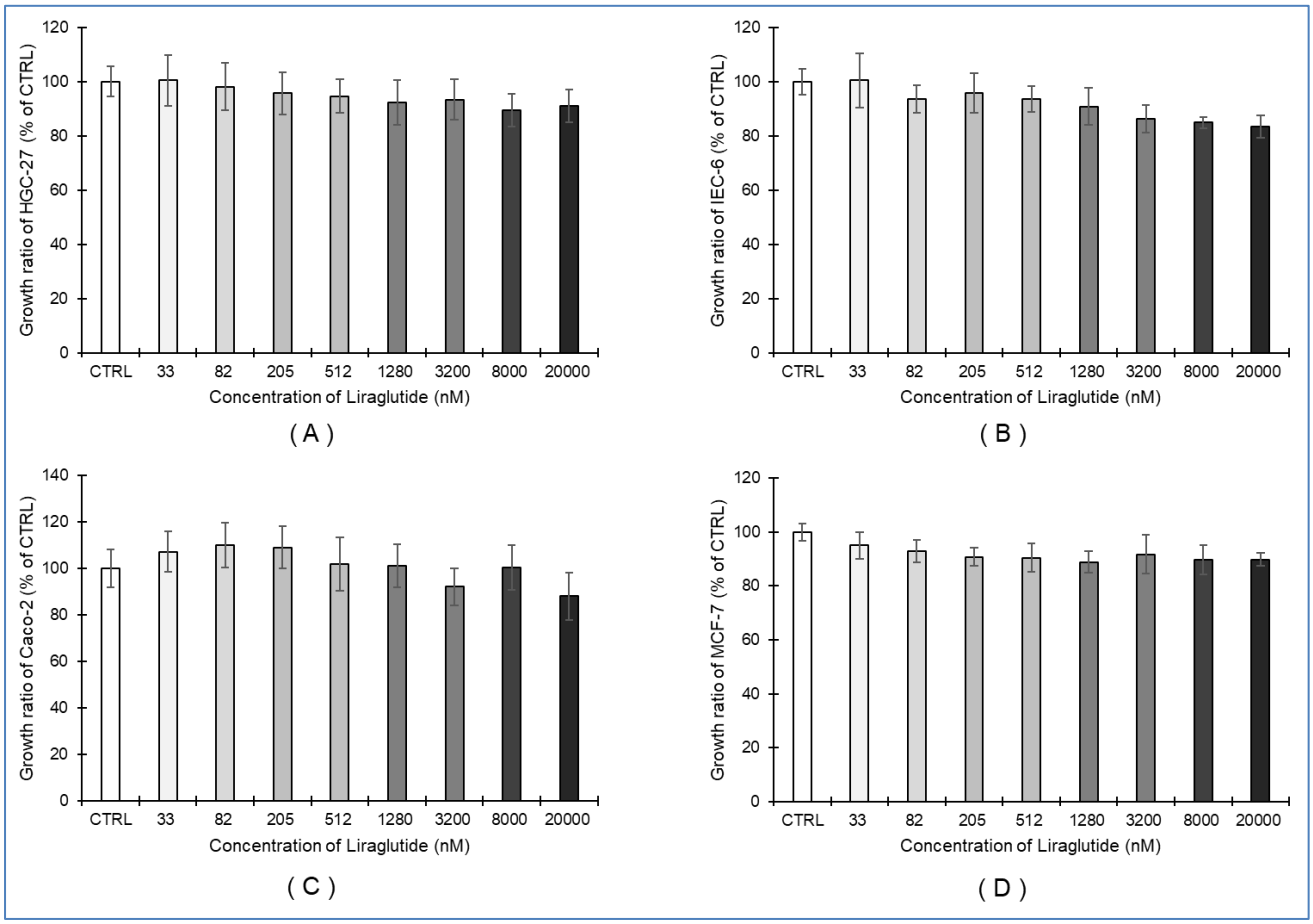

Fig-2: Effects of Liraglutide on the proliferation of HGC-27 (A), IEC-6 (B), Caco-2 (C), and MCF-7 (D) cells. Data represent means and S.D. (n = 6 for each condition)

A previous study reported that 50 μM of Liraglutide inhibited the growth of MCF-7 cells [22]; however, another study showed that it promoted the growth of breast cancer cells [16]. Therefore, the effects of Liraglutide on the proliferation of cultured cells, including MCF-7, remain controversial. Further studies are needed on GLP-1 receptor agonists and the expression levels of the GLP-1 receptor.

Effects of Liraglutide on H_2O_2-induced Cytotoxicity

Aside from glycemic control, GLP-1 receptor agonists have been reported to exert protective effects against oxidative stress [23, 24]. Therefore, the protective effects of Liraglutide against oxidative stress in gastrointestinal epithelial cells were investigated in the present study. The results obtained showed that Liraglutide did not attenuate reductions in cell viability by H_2O_2 in the three gastrointestinal epithelial cell lines. The protective effects of Liraglutide against oxidative stress were only observed in Caco-2 cells, but were not significant (Fig. 3C). Similarly, Liraglutide did not inhibit the expression of Caspase-9, an indicator of apoptosis, or HIF-1α, an indicator of oxidative stress (data not shown). The results shown in Fig. 3 were obtained at a Liraglutide concentration of 1000 nM; however, no significant changes were observed at concentrations of 125 ~ 2000 nM. Therefore, Liraglutide did not exert protective effects against H_2O_2-induced oxidative stress in gastrointestinal epithelial cells (Fig. 3). Since Liraglutide did not attenuate H_2O_2-induced oxidative stress in cells with high or low GLP-1 receptor expression levels, further studies, including those on the mechanisms underlying its protective effects, are needed.

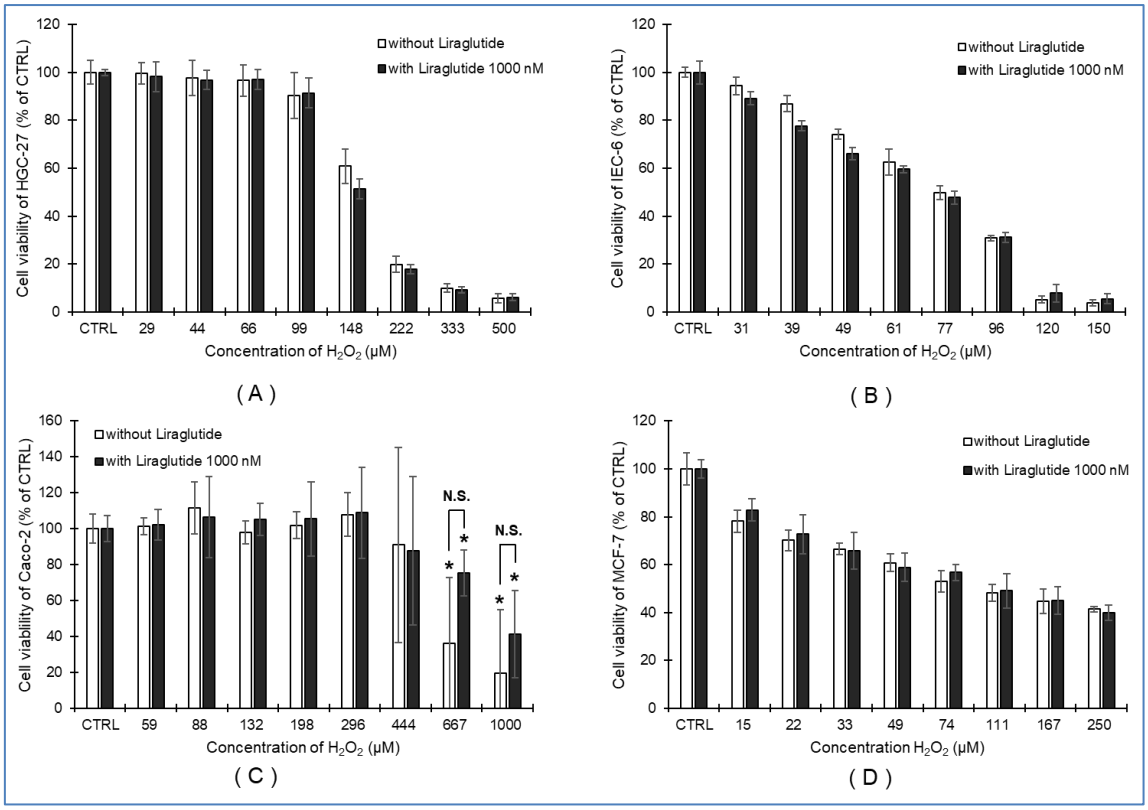

Fig-3: Effects of Liraglutide on H_2O_2-induced oxidative stress in HGC-27 (A), IEC-6 (B), Caco-2 (C), and MCF-7 (D) cells. Data represent means and S.D. (n = 4 for each condition). N.S.: not significant, *: P <0.05 significantly different from each CTRL condition.

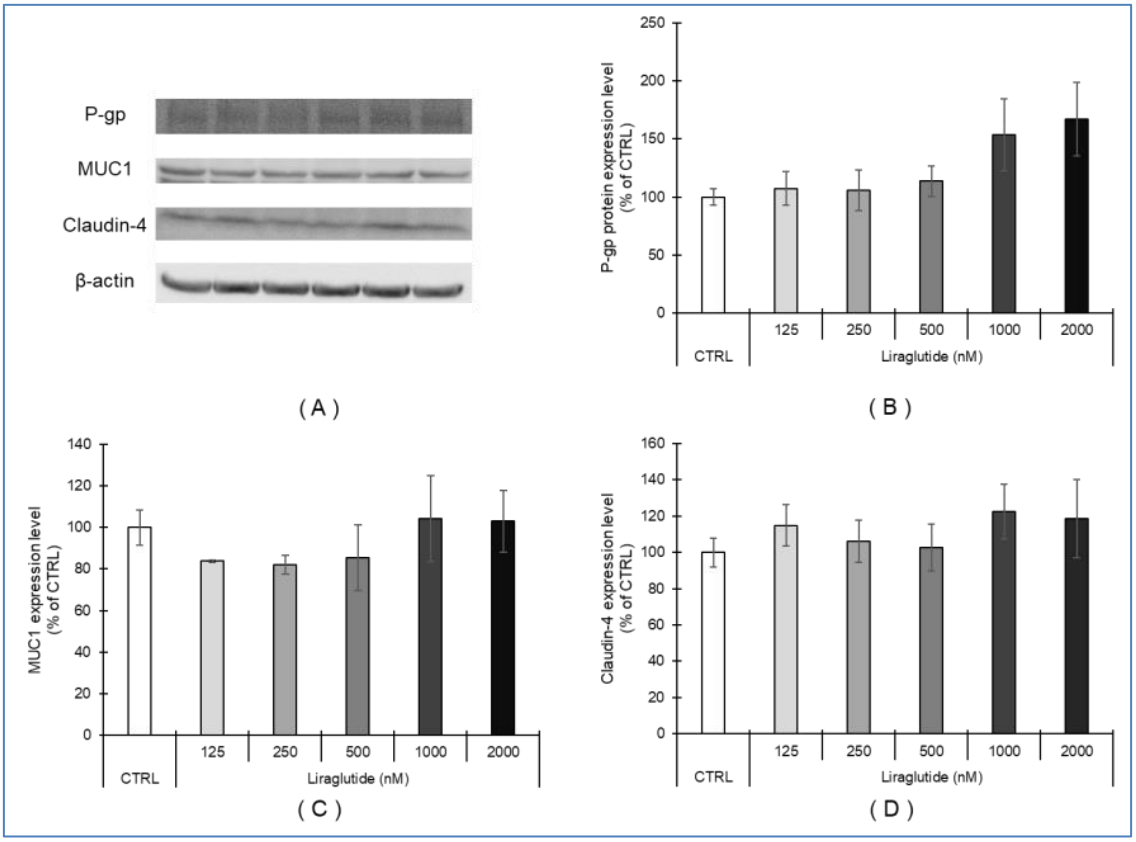

Fig-4: Effects of Liraglutide on protein expression levels of absorption-regulating factors in Caco-2 cells. (A): Typical bands for Western blotting, (B): P-gp, (C): MUC1, and (D): claudin-4. Data represent means and S.D. (n = 4 for each condition)

Effects of Liraglutide on Expression Levels of Absorption-regulating Factors

Since patients with diabetes require long-term (chronic) medication with diabetes drugs, they have many opportunities to be taken in combination with other drugs. A more detailed understanding of the changes induced in absorption-regulating factors under chronic treatment with GLP-1 receptor agonists is important. Therefore, the present study investigated the effects of Liraglutide on absorption-regulating factors in Caco-2 cells, which are commonly used in drug permeation experiments.

P-glycoprotein (P-gp; ATP-binding cassette (ABC) B1), an important factor influencing drug-drug interactions, MUC1, a component of the unstirred water layer, which is a rate-limiting factor in passive diffusion for lipophobic drugs, and claudin-4, a component of the tight junctions that regulate membrane permeation via the paracellular route, were examined as absorption-regulating factors in the present study.

The results obtained showed no significant changes in the expression levels of these proteins (Fig. 4); however, a slight increase was noted in P-gp protein expression levels that were dependent on the concentration of Liraglutide (Fig. 4B).

In the human breast adenocarcinoma cell line MDA-MB-231, occludin expression levels were reduced [25], while ABC transporter expression was increased by Liraglutide [26]. Although the gastrointestinal luminal approach was not used, previous findings demonstrated that a GLP-1 receptor agonist inhibited the gastric emptying rate by suppressing gastric peristalsis and increasing pyloric tone contractions [27], and also delayed duodenal-small intestinal emptying time [28]. Therefore, significant changes in the expression of these proteins may occur with a long-term exposure to Liraglutide.

CONCLUSION

In this study, the effects of Liraglutide, a GLP-1 receptor agonist, on 3 gastrointestinal epithelial cell lines were examined assuming after ingestion of orally administered formulations of GLP-1 receptor agonists. Based on the absence of any significant changes in any of the cell lines tested, even at high concentrations of Liraglutide, there were no significant effects at the cellular level. However, since the present results were contradictory to previous findings, further studies using other conditions and cells are warranted.

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CONFLICT OF INTEREST (COI)

The authors declare no conflict of interest.

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