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Research Paper

Enhancement by adrenaline of ginsenoside Rg1 transport in Caco-2 cells and oral absorption in rats

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Abstract

Objectives The purpose of this research was to evaluate the ability of adrenaline (epinephrine) to stimulate the uptake of ginsenoside Rg1 (Rg1) by Caco-2 cells.

Methods Rg1 uptake was measured using Caco-2 cell monolayers. The Rg1 uptake medium with adrenaline at different concentrations was added to each well and incubated for different time intervals. Adrenergic antagonists such as phentolamine and propranolol were added to the incubation medium to investigate their effects on Rg1 uptake. The Rg1 concentration in the monolayers was determined by high-performance liquid chromatography. Transport of Rg1 across Caco-2 cells was also studied and an oral bioavailability study of Rg1 was carried out in rats.

Key findings The incubation medium with adrenaline remarkably increased the amount of Rg1 uptake by Caco-2 cells. Adrenaline-induced Rg1 transport increased in a dose- and time-dependent manner. The effect of adrenergic antagonists on adrenaline-induced uptake of Rg1 was investigated and it was found that the enhancement effect was attenuated by the co-treatment with propranolol but not phentolamine. The transport amount of Rg1 by Caco-2 cells increased in response to 1 mM adrenaline, isoproterenol or salbutamol. In contrast, 1 mM phenylephrine had no effect on Rg1 transport in Caco-2 cells. The effect of adrenaline on the absorption of Rg1 was further investigated *in vivo* in rats. The co-administration with adrenaline in rats showed that the oral bioavailability was increased remarkably relative to the aqueous solution. The area under the plasma concentration–time curve of Rg1 after co-administration with 1 mM adrenaline was 79.1 ± 31.04 μg/ml/h compared with 2.81 ± 1.13 μg/ml/h for its aqueous solution.

Conclusions Adrenaline is effective for the stimulation of intestinal absorption of Rg1 and the enhanced absorption is mediated mainly by the interaction of adrenaline with β_2-adrenoceptors.

Keywords absorption enhancer; Caco-2 cells; adrenaline (epinephrine); ginsenoside Rg1; oral bio-availability

Introduction

Panax ginseng C.A. Meyer (Araliaceae) has been used as a tonic medicine to enhance stamina and relieve fatigue and stress for many centuries in Asian countries. Ginsenosides, the most important components isolated from *P. ginseng*, exhibit a variety of biological activities. In particular, ginsenoside Rg1 (Rg1) has been shown in recent years to possess a number of pharmacological effects, such as immune-modulating activities, stimulatory effect on the central nervous system, suppression of hepatoma cell growth and protective effect on experimental myocardial injury.[1–7]

We have studied the pharmacokinetics of Rg1 in rats and have demonstrated limited absorption from the digestive tract.[8] The low oral bioavailability of Rg1 could be due to its low membrane permeability,[9] decomposition in the stomach,[9,10] metabolism in the intestine, elimination in the liver[11] and tendency for self-micelle formation.[12] In order to achieve better efficacy, it is important to improve the oral absorption of Rg1. However, very few studies have elucidated the transport mechanism and enhancement of Rg1.

Numerous classes of compounds are reported to improve the transport of such incompletely absorbed dugs.[13] These enhancers may act as the openers of the transcellular

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or paracellular pathways, or both.[14] Although they have been demonstrated to increase drug uptake, intestinal toxicity induced by these enhancers cannot be excluded.[13,15] Therefore, a lot of attention has been focused on the application of physiological enhancers such as nitric oxide due to its optimal efficacy and low toxicity.[16–18]

Adrenaline (epinephrine), a physiological substance, has been used as a useful absorption enhancer. The perfusion of rat small intestinal lumen with adrenaline (0.1 mM) resulted in a significant increase in the amount of benzylpenicillin transported from the mucosal to the serosal side.[19] Adrenaline has also been reported to have a stimulatory effect on the transport of dextran with a molecular mass of 4000 Da in rat jejunum with no toxic effect.[20] Both dextran and Rg1 are hydrophilic compounds. Dextran is a complex, branched polysaccharide made of many glucose molecules, while, structurally, Rg1 belongs to the glucosides.

In the present study, we investigated the absorption enhancing effects of adrenaline on the uptake and transport of Rg1 by Caco-2 cells. Caco-2 cells have been widely used to study the transport kinetics of different drugs and predict drug absorption.[21,22] Caco-2 cells are human in origin and present many features of absorptive intestinal cells such as some intestinal receptors (e.g. insulin receptor)[23] and carrier-mediated transport systems.[24] In order to substantiate Caco-2 cell results, we further examined the efficacy of adrenaline on the in-vivo oral absorption of Rg1 in rats.

Materials and Methods

Materials

Rg1 was purchased from Kunming Phytopharmaceutical Co. Ltd (Yunnan, China). The purity was 98.1%, which was verified by high-performance liquid chromatography (HPLC). The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, US). Cell culture media and reagents including fetal bovine serum were purchased from Gibco (Grand Island, NY, US). Millicell cell culture inserts and Millicell-ERS were purchased from Millipore (Bedford, MA, US). Adrenaline, phentolamine, propranolol, phenylephrine and dobutamine hydrochloride were purchased from Sigma Chemical Co. (St Louis, MO, US). Isoproterenol hydrochloride was purchased from Aldrich Chemical Co. Milwaukee, WI, US). Acetonitrile (Merck, Darmstadt, Germany) and methanol (Sandong Yuwang Industrial and Commercial Co., Ltd (Shandong, China)) were of HPLC grade. All the other chemicals were of reagent grade.

Cell culture

Caco-2 cells at passages 35–50 were used in the experiments; they were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamate, 1% non-essential amino acids and 1% penicillin–streptomycin solution at 37°C in 75-cm^2 flasks in a humidified air/5% CO_2 atmosphere. After being harvested with trypsin-ethylenediamine tetraacetic acid, cells in suspension (5×10^4 cells/ml) were seeded onto 24-well plates for Rg1 uptake studies (400 μl/well) and on permeable polycarbonate inserts (0.6 cm^2, 0.40 μm pore size) in 24-well plates for transport studies (400 μl cell suspension in the apical compartment and 600 μl culture medium in the basolateral compartment). Medium was replaced every 2 days thereafter. Cells were cultured for 14 days for uptake studies and 20–22 days for transepithelial transport studies after seeding. The transepithelial electrical resistance (TEER) of the Caco-2 cells was examined routinely using the Millicell-ERS apparatus to assess the integrity of monolayers grown on the permeable membrane in the transepithelial transport studies. The monolayers were used when TEER exceeded 600 Ω cm^2.

Uptake studies

Rg1 uptake was measured using monolayers grown in 24-well plates. The uptake medium consisted of Hanks' balanced salt solution (HBSS) with 1.26 mM $CaCl_2$, 0.9 mM $MgCl_2$ and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (adjusted to pH 7.4 with 1 mol/l HCl). For Rg1 uptake assays, cells were washed 3 times with HBSS at pH 7.4. Rg1 uptake medium (1 mg/ml) with adrenaline at different concentrations was added to each well and incubated for different time intervals (15 and 30 min, 1 and 2 h) at 37°C. Adrenergic antagonists such as phentolamine and propranolol were added to the incubation medium of the cells to investigate their effects on Rg1 uptake. At the end of the incubation period, the medium was aspirated and the cells were carefully rinsed with ice-cold HBSS. Each Caco-2 cell monolayer was collected with 0.5 ml HBSS and frozen (at −70°C) then thawed (at room temperature) 3 times. The cells were then lysed ultrasonically. The samples were mixed with isometric methanol by vortexing for 30 s, and centrifuged at 5645 *g* for 10 min. The supernatants were collected and 50 μl was used to determine the Rg1 concentration using the HPLC method described below. Results were expressed as μg of Rg1/mg of protein. The protein contents of the cells were determined by Coomassie staining.

Transport studies

Transport of Rg1 across the Caco-2 cells was studied with monolayers 21–24 days after seeding. Before the experiments, the monolayers were washed twice with HBSS. The monolayers were preincubated at 37°C for 15 min. HBSS solution on both sides of the cells was removed by aspiration. Then, 400 μl incubation medium of Rg1 with or without different adrenergic agonists were added to the apical compartments. HBSS (600 μl, pH 7.4) was added on the basolateral side. The monolayers were incubated at 37°C. Samples (200 μl) were taken from the basolateral compartments at 30 min, 1 and 2 h, followed by an immediate replacement with HBSS. A total of 50 μl was used to determine the Rg1 concentration by HPLC (described below). All experiments were conducted in triplicate. At the end of the experiment, the effect of adrenergic agonists on the opening of intestinal tight junctions was checked by TEER measurement.

The apparent permeability coefficient (P_{app}) was determined according to the equation: $P_{app} = (dQ/dt)/(A \times C_0)$, where dQ/dt is the drug permeation rate (μg/s), A is the surface area of epithelium (cm^2), and C_0 is the initial concentration in the donor compartment ($\mu g/cm^3$).

MTT toxicity assay

MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenase in living but not dead cells to give a dark blue product.[25,26] Calibration experiments showed that the dehydrogenase activity was linearly correlated with cell number over the range of 5000–50 000 cells per well (data not shown). In our experiments, Caco-2 cells were seeded at a density of 1×10^4 cells/well in 96-well plates in the same medium used for culture in cell culture flasks. The cells were grown at 37°C in a 5% CO_2 atmosphere for 48 h before the cell viability assays. The culture medium was then replaced by 100 μl of HBSS containing the test solutions or controls at a predetermined concentration range. After 2 h of cell incubation with the formulations, 20 μl of the MTT solution (5 mg/ml in PBS, pH 7.3) was added to each well. In 4 h, the medium was removed. Any formazan crystals generated during that process were solubilised with 150 μl of DMSO. The absorbance of formazan, a metabolite of MTT, was measured at a wavelength of 490 nm using a microplate reader.

Oral bioavailability study of ginsenoside Rg1 in rats

Sprague–Dawley rats, 220–250 g, were obtained from the Jiangsu animal breeding center, Nanjing, China. The animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University. The rats were fasted for 20 h before the experiment.

Rg1 aqueous solution (40 mg/ml) with or without adrenaline (4 mg/kg) was administered orally at a dose of 200 mg/kg to fasted rats with free access to water. Each group had six rats. Blood samples were collected from the eye socket at 5, 15, 30, 45 and 60 min, 1.5, 2, 4, 8, 12 and 24 h after administration under light ether anaesthesia.

Determination of ginsenoside Rg1 by HPLC

Blood samples were pretreated with solid phase extraction cartridges (OASIS, Waters, US). HPLC was conducted with an Agilent 1100 liquid chromatography system comprising a binary solvent delivery system, an autosampling device and UV detector. A Diamonsil C18 reverse phase column (5 μm, 4.6 i.d. × 250 mm; Dikma, Beijing, China) was applied and maintained at 40°C. UV absorption was measured at 203 nm. The mobile phase was composed of acetonitrile–water (25 : 75, v/v) and the flow rate was 1 ml/min.

Statistical analysis

All values are the mean ± SD for any group of samples. The statistical differences between means were determined by the Student's test and comparisons among more than three groups were performed by using one-way analysis of variance, Kruskal–Wallis test, Mann Whitney *U*-test and Student–Newman–Keuls test. $P < 0.05$ was considered to be significant.

Results

Effect of adrenaline on uptake of ginsenoside Rg1 in Caco-2 cells

To determine the effective concentration in response to adrenaline treatment for Rg1 uptake, Caco-2 cells were incubated with different concentrations of adrenaline (0.01–10 mM) for 2 h. As shown in Figure 1, the intracellular amount of Rg1 at 37°C increased with the increase in the concentration of adrenaline from 0.01 to 10 mM ($P < 0.01$).

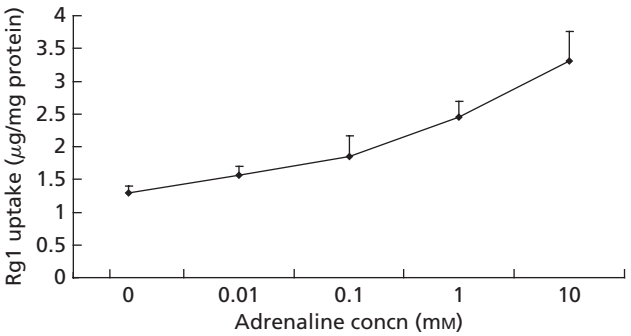

Figure 1 Effect of adrenaline on ginsenoside Rg1 uptake by Caco-2 cells. Concentration-dependent uptake of ginsenoside Rg1 by Caco-2 cells incubated with adrenaline. Each point represents the mean ± SD of at least three independent samples.

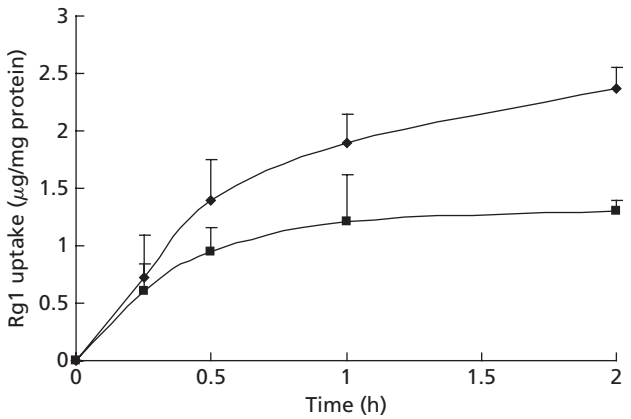

Figure 2 Time course of ginsenoside Rg1 uptake in Caco-2 cells. Time course of ginsenoside Rg1 uptake in Caco-2 cells incubated with (♦) or without (■) 1 mM adrenaline at 37°C. Results are expressed as means ± SD ($n = 4$).

We examined the time-dependent effect of 1 mM adrenaline on Rg1 uptake. As shown in Figure 2, the uptake amount of Rg1 without adrenaline (control) had almost reached a steady state after the first 60 min. During the 2 h incubation with adrenaline, the uptake amount of Rg1 into Caco-2 cells gradually increased with time ($P < 0.05$). Adrenaline significantly enhanced Rg1 uptake 30 min after treatment ($P < 0.05$). The uptake amount of Rg1 at 2 h was 2.37 μg/mg protein in the presence of adrenaline, 1.8-fold compared with control ($P < 0.001$).

The incubation medium with the addition of adrenaline remarkably increased the amount of Rg1 uptake by Caco-2 cells. Adrenaline-induced Rg1 transport increased in a dose-dependent and time-dependent manner.

Effects of adrenergic antagonists on adrenaline-induced uptake of ginsenoside Rg1 by Caco-2 cells

The effects of adrenergic agonists on adrenaline-induced uptake of Rg1 were investigated. As shown in Table 1, 10 μM

Table 1 Effects of adrenergic antagonists on adrenaline-induced uptake of ginsenoside Rg1 by Caco-2 cells

Treatment	Ginsenoside Rg1 uptake (μg/mg protein) ($\bar{x}$ ± SD)
Control	1.30 ± 0.10
Adrenaline	1.57 ± 0.13*
Phentolamine	1.33 ± 0.13
Adrenaline + phentolamine	1.44 ± 0.18
Propranolol	1.25 ± 0.09
Adrenaline + propranolol	1.18 ± 0.04†††

Each value represents the mean ± SD (n = 4). *P < 0.05, significantly different compared with the control value. $^{\dagger\dagger\dagger}P$ < 0.001, significantly different compared with adrenaline.

adrenaline significantly enhanced the cellular uptake of Rg1: 1.567 ± 0.121 μg/mg protein per min with adrenaline compared with 1.297 ± 0.099 μg/mg protein per min without adrenaline (P < 0.05). Based on the Kruskal–Wallis test, the effects of adrenergic antagonists on adrenaline-induced uptake of Rg1 were significantly different (P < 0.05). A non-specific β-adrenergic receptor antagonist (propranolol) and a non-specific α-adrenergic receptor antagonist (phentolamine) were applied to investigate the effects of adrenergic antagonists on adrenaline-induced uptake of Rg1. Propranolol or phentolamine used alone did not influence the uptake of Rg1. The addition of propranolol at a concentration of 100 μM blocked the effect of adrenaline on uptake of Rg1 (P < 0.001). However, the addition of phentolamine (100 μM) did not influence the uptake of Rg1.

Effects of adrenergic agonists on ginsenoside Rg1 transport by Caco-2 cells

The effect of adrenergic agonists on the transport of Rg1 by Caco-2 cells are shown in Table 2. According to the Kruskal–Wallis test, the effects of adrenergic agonists were significantly different (P < 0.01). The addition of 1 mM adrenaline to the apical medium markedly increased the amount of Rg1 transported to the basolateral side. The apparent permeability coefficient (P_{app}) (apical to basolateral) for Rg1 by treatment with adrenaline was 8.35 ± 2.63 × 10^{-7} cm/s, which was significantly greater than the control 1.34 ± 0.25 × 10^{-7} cm/s (P < 0.05). The β-adrenergic agonist, isoproterenol, stimulated Rg1 transport by Caco-2 cells, and the P_{app} (apical to basolateral) of Rg1 was determined to be 11.83 ± 6.15 × 10^{-7} cm/s (P < 0.05). Another β_2-adrenergic agonist salbutamol also had the same stimulatory effects and the amount of Rg1 transported to the basolateral side was 4.27-times that with the control. Dobutamine (β_1-adrenergic agonist) did not show much stimulating effect.

Table 2 Effects of adrenergic agonists on adrenaline-induced ginsenoside Rg1 transport by Caco-2 cells

Treatment	P_{app} of Rg1 ($\times 10^{-7}$cm/s)
Control	1.34 ± 0.25
Adrenaline (1 mM)	8.35 ± 2.63*
Phenylephrine (1 mM)	0.99 ± 0.36
Isoproterenol (1 mM)	11.83 ± 6.15*
Dobutamine (1 mM)	2.48 ± 1.80
Salbutamol (1 mM)	5.72 ± 3.63

P_{app}, apparent permeability coefficient. Each value represents the mean ± SD. *P < 0.05, significantly different compared with the control value.

The effect of adrenergic agonists on the opening of intestinal tight junctions was assessed by measuring the TEER at the beginning and end of the experiment. The TEER of the test groups was not significantly different compared with that of the control group (data not shown).

MTT toxicity assay

The effect of adrenaline on mitochondrial dehydrogenase activity was determined using the MTT method. The maximum concentration of adrenaline used for our study was 10 mM, which was not toxic towards the Caco-2 cells. The absorption-enhancing effect caused no perturbation of the cell membrane.

Effect of adrenaline on rat oral absorption of ginsenoside Rg1 *in vivo*

The plasma concentration profiles of Rg1 in rat after oral administration with and without adrenaline at a dose of 200 mg/kg are shown in Figure 3. After co-administration with adrenaline, the concentration–time profile of Rg1 was significantly different compared with that of the aqueous solution (control) (P < 0.05). Without the presence of an enhancer, Rg1 was detected in plasma 5 min after administration and reached a maximum level of 4.46 μg/ml at 15 min. Rg1 was practically undetectable 4 h later. A significantly greater plasma level of Rg1 was achieved when co-administered with adrenaline, compared with the control group. Bi-absorption peaks were evident with much longer residence time and a greater extent of absorption. The area under the plasma concentration–time curve (AUC) of Rg1 after co-administration with adrenaline (1 mM) was 79.1 ± 31.04 μg/ml per h, compared with 2.81 ± 1.13 μg/ml per h for its aqueous solution (P < 0.001). It appeared that adrenaline was effective in enhancing the absorption of Rg1

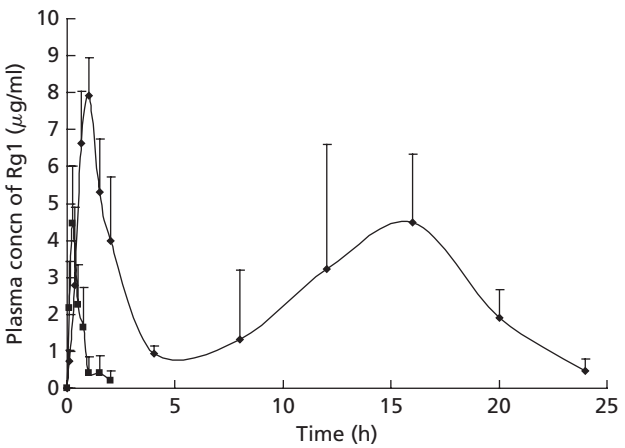

Figure 3 Plasma concentration profiles of ginsenoside Rg1. Plasma concentration profiles of ginsenoside Rg1 (200 mg/kg) after oral administration to rats with (♦) and without (■) adrenaline. Each point represents the mean ± SD of six animals.

in vivo despite the existence of the metabolic enzyme in the gastrointestinal tract.

Discussion

Effect of adrenaline on uptake of Rg1 in Caco-2 cells

The uptake amount of Rg1 by Caco-2 cells increased remarkably when incubated with adrenaline. The amount of adrenaline-induced Rg1 transport also increased in a dose-dependent and time-dependent manner. The intestinal mucosa is extensively innervated by adrenergic fibres. Adrenoceptors identified in epithelial cells[27] are classified as α-adrenoceptors and β-adrenoceptors. α_2-Adrenoceptors have been detected in the intestinal mucosa.[28,29] The β-adrenoceptors are located in the basolateral membrane of epithelial cells.[30] It appears that adrenaline either interacts directly with the respective adrenoceptor located in the epithelial cells or affects these cells indirectly via the enteric nervous system.

Effects of adrenergic antagonists on adrenaline-induced uptake of Rg1

To determine which adrenergic receptor subtype mediates the stimulation of adrenaline-induced uptake, various adrenergic receptor antagonists were applied during the uptake. The enhancement effect was attenuated by the co-treatment with propranolol, but not phentolamine. This result suggests that adrenaline-induced uptake of Rg1 was mediated by β-adrenoceptors.

Effects of adrenergic agonists on Rg1 transport by Caco-2 cells

The effects of adrenergic agonists on transport of Rg1 by Caco-2 cells were investigated. The transport amount of Rg1 by Caco-2 cells increased in response to adrenaline, isoproterenol or salbutamol at a concentration of 1 mM. In contrast, 1 mM phenylephrine had no effect on Rg1 transport in Caco-2 cells. This result implied that the Rg1 transport induced by adrenaline was mediated mainly through β_2-adrenoceptors. Moreover, no significant reduction in TEER indicated that the increased permeability of the monolayers was not mediated via the paracellular route.

Effect of adrenaline on rat oral absorption of Rg1 *in vivo*

The effect of adrenaline on the absorption of Rg1 was further investigated *in vivo* in rats. The co-administration with adrenaline in rats showed that the oral bioavailability was increased remarkably relative to the aqueous solution. The $AUC_{0\to\infty}$ after administration of adrenaline (4 mg/kg) was 28.19-times that of the control group.

In the present study, the concentration–time profile of Rg1 exhibited double peaks after oral co-administration with adrenaline *in vivo*. The investigation carried out by Odani *et al.*[8] demonstrated that the cumulative biliary excretion of Rg1 within 4 h was 57 ± 1.7% of the dose after intravenous administration. The study indicated that Rg1 might be extensively eliminated into the rat bile and probably re-absorbed by the small intestine. However, due to the poor absorption of Rg1 from the gastrointestinal tract, the re-absorption phenomenon could not be observed. The pharmacokinetic data obtained in the presence of adrenaline revealed the presence of biliary excretion and potential for enterohepatic cycling. Moreover, it took time for adrenaline to interact with adrenergic receptors in epithelial cells, which might result in the observed delay in the time to reach the maximum plasma level. The maximum concentration (C_{max}) was postponed from 15 min to 1 h. A similar delay was observed in Caco-2 cells uptake. The uptake of Rg1 was significantly enhanced 30 min after treatment, however no statistically significant difference was observed at 15 min.

The mechanism of adrenaline enhancement has been investigated by various research groups. It has been found that adrenaline regulates many types of transporter systems by interacting with α_2- or β-adrenoceptors. Adrenaline increases the activity of the Na^+/H^+ antiporter by acting at α_2-adrenoceptors in glioma cells[31] and increases that of the $Na^+/K^+/2Cl^-$ cotransporter through β-adrenoceptor activation in tracheal epithelial cells.[32] In contrast, adrenaline inhibits the $Na^+/K^+/2Cl^-$ cotransporter via β-adrenoceptor activation in lymphocytes.[33] This neurotransmitter also increases the amount of the Na^+/glucose cotransporter (SGLT1) in the basolateral membrane of intestinal epithelial cells by interacting with β-adrenoceptors in the basolateral membrane.[30] For Rg1, the mechanism of absorption enhancement by adrenaline is mediated mainly via β_2-adrenoceptors, as shown in this study. These findings indicate that some active transporter might be involved.

Conclusions

We found that adrenaline may be an effective absorption enhancer for Rg1 without causing any significant cytotoxic effect on the Caco-2 cells. The absorption enhancement is mediated mainly by the interaction of adrenaline with β_2-adrenoceptors. After co-administration with adrenaline, the oral bioavailability was enhanced remarkably relative to the aqueous solution. These findings indicate that the application of adrenaline may be a new strategy to increase the intestinal absorption of drugs.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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