Glucagon-like peptide-1 analogue exendin-4 modulates serotonin transporter expression in intestinal epithelial cells

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Abstract. Serotonin-selective reuptake transporter (SERT) regulates extracellular availability of serotonin (5-hydroxytryptamine; 5-HT) and participates in the pathogenesis of functional disorders. Colonic SERT expression is decreased in colonic sensitized rats, and the glucagon-like peptide-1 analogue, exendin-4, reduces visceral hypersensitivity by decreasing 5-HT levels and increasing SERT expression. The present *in vitro* study aimed to further investigate the effects of exendin-4 on SERT expression, and to examine the role of GLP-1 and its receptor in the regulation of 5-HT. SERT mRNA and protein expression levels were detected by reverse transcription-quantitative PCR and western blotting. A $^{[3H]}$-5-HT reuptake experiment was performed in IEC-6 rat intestinal epithelial cells treated with exendin-4. Effects on the adenosine cyclophosphate (AC)/PKA pathway were examined by variously treating cells with the AC activator forskolin, the protein kinase A (PKA) inhibitor H89 and the AC inhibitor SQ22536. Exendin-4 treatment upregulated SERT expression and enhanced 5-HT reuptake in IEC-6 cells. Also, PKA activity in IEC-6 cells was increased by both exendin-4 and forskolin, whereas these effects were abolished by the pre-treatment of exendin-9, which is a GLP-1R inhibitor, SQ22536 and H89. In conclusion, exendin-4 may be associated with the upregulation of SERT expression via the AC/PKA signaling pathway.

Introduction

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal disorder with a worldwide prevalence of 5-20% (1,2); it is characterized by chronic abdominal pain and discomfort, as well as changes in bowel habits. The pathogenic mechanisms of IBS remain unknown; however, accumulating evidence has indicated that abnormal gastrointestinal motility and visceral hypersensitivity are two important pathophysiologic features of IBS (3,4), and that the latter contributes to abdominal pain in patients. Currently, there is no satisfactory management method available for patients with IBS. Hellström *et al* (5) demonstrated that the glucagon-like peptide-1 (GLP-1) analogue, ROSE-010, could effectively relieve IBS pain exhibited by patients. However, the underlying mechanisms governing this remain poorly understood. Recent research has indicated that the GLP-1 analogue liraglutide alters the visceral sensation in patients with IBS (6), indicating that GLP-1 may be useful as a treatment for this disease.

GLP-1 is a common incretin hormone that is released from intestinal L-cells in response to nutrient ingestion (7,8). GLP-1 can enhance insulin secretion, delay gastric emptying, inhibit motility and exert antispasmodic effects (9). A previous study indicated that exendin-4, a GLP-1 analogue, reduced visceral hypersensitivity by increasing serotonin-selective reuptake transporter (SERT) expression, a consequence of decreasing serotonin (5-hydroxytryptamine; 5-HT) levels (10). The short half-life of GLP-1 presents a considerable barrier to its therapeutic use, whereas exendin-4, which has 53% homology with GLP-1, exhibits a longer half time and can be used to mimic the effects of GLP-1 (11). GLP-1 exerts biological functions by binding to its specific receptor, GLP-1R, in the stomach, intestine and brain (11,12). GLP-1 also stimulates cyclic adenosine monophosphate (cAMP) formation and subsequently induces protein kinase A (PKA) activity by binding to GLP-1R (13). SERT expression can be regulated by a variety of stimuli, including cAMP (14,15). However, the GLP-1/GLP-1R/cAMP signaling pathway in intestinal epithelial cells has, to the best of our knowledge, not yet been investigated.

SERT is expressed in intestinal epithelial cells, and this expression is significantly decreased in the colon and rectum of patients with IBS (16,17); the removal of 5-HT by SERT is important in inhibiting 5-HT activity (18). 5-HT is a neurotransmitter that has been examined in rodents and humans, and is expressed at high levels in the intestinal mucosa of patients with IBS with constipation (IBS-C) (19). 5-HT is released from enterochromaffin cells in response to mucosal stimuli, and it can initiate motor reflexes (20) and visceral sensation (21); 5-HT can be inactivated by SERT-mediated uptake into enterocytes or neurons (18). Abnormal serotonergic signaling can contribute to visceral hypersensitivity in IBS, and a number of serotonergic drugs can relieve IBS symptoms (22).

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In a previous study, immunochemistry results demonstrated that GLP-1R expression was located in the colonic mucosa layer in rodent models of IBS, especially in IBS-C models (23). Furthermore, it has been shown that in colonic sensitized rats, SERT expression was decreased, and exendin-4 treatment reduced visceral hypersensitivity by increasing SERT expression and decreasing 5-HT content (10). These results demonstrated that GLP-1 binding to GLP-1R may be associated with SERT expression and 5-HT content, a consequence of the formation of visceral hypersensitivity in IBS models. However, the underlying intracellular mechanisms governing this are yet to be determined. The current study aimed to investigate whether the GLP-1 analogue exendin-4 was able to modulate SERT expression via the adenosine cyclophosphate (AC)/PKA/SERT signaling pathway in IEC-6 rat intestinal epithelial cells.

Materials and methods

Cell culture. Normal rat intestinal epithelial cell line IEC-6 were cultured in completed DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) (v/v), 2 mM L-glutamine, 1% antibiotic (v/v) solution containing penicillin G (10,000 U/ml) and streptomycin (10,000 U/ml), 1.5 g/l $NaHCO_3$, 2 g/l HEPES and 0.01 mg/ml insulin, and maintained in a 37˚C homothermal incubator with a 5% CO_2 atmosphere. IEC-6 cells were seeded into six-well plates and cultured for 48 h (to 80% confluence) in complete DMEM. Cells were then cultured in serum-free DMEM for 12 h, and then exposed to exendin-4 at different concentrations (0, 0.1, 1, 10 and 100 nM) for 12 h at 37˚C to select the optimal concentration, as described previously (24). Subsequent experiments were conducted at 37˚C for a number of time periods (0, 3, 6, 12 and 24 h). In certain experiments, the GLP-1R antagonist exendin-9 (10 μM; Sigma-Aldrich; Merck KGaA) was used 1 h prior to exendin-4 treatment at 37˚C. The IEC-6 cells were pre-stimulated with H89 (10 μM; Beyotime Institute of Biotechnology) and SQ22536 (10 μM; Cayman Chemical Company) for 30 min at 37˚C with or without exendin-4 treatment. Forskolin (5 μM; Cayman Chemical Company) was administrated for 12 h at 37˚C with or without SQ22536 pre-treatment in IEC-6 cells. All experiments were performed in triplicate.

PKA activity assay. IEC-6 cells were lysed in cold PKA extraction buffer containing 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol and 50-fold diluted proteinase inhibitor cocktail (25). The homogenates were centrifuged at 20,000 x g for 5 min at 4˚C, and PKA activity in the supernatants was subsequently assessed using a non-radioactive PKA kinase assay kit, Type I (ImmuneChem Pharmaceuticals, Inc.), according to the manufacturer's protocols.

$^{[3H]}$5-HT re-uptake. The serum-free medium was removed by aspiration, and the cells were washed with 1 ml of Krebs-Ringer's (KRH) buffer (130 mM NaCl; 1.3 mM KCl; 2.2 mM $CaCl_2$; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 1.8 g/l glucose and 10 mM HEPES, pH 7.4). A total of 1 ml KRH buffer containing 100 μM pargyline and 100 μM ascorbic acid with or without exendin-4 was subsequently added to IEC-6 cells for 10 min in a 37˚C chamber with a 5% CO_2 atmosphere. The $^{[3H]}$5-HT reuptake assays were initiated by adding 100 nM $^{[3H]}$5-HT (27.9 Ci/mmol; NET498; PerkinElmer, Inc.) and the cells were then incubated in a 37˚C chamber for a subsequent 10 min. The cells were washed rapidly with cold KRH buffer to terminate the reuptake assays and solubilized in 600 μl 1% Triton. The cell lysate (50 μl) was mixed in OptiPhase Supermix scintillation mixture for radioactivity counting (Wallac Liquid Scintillation Counter; PerkinElmer, Inc.). Non-specific uptake $^{[3H]}$5-HT was defined using 100 μM paroxetine (a SERT inhibitor; Enzo Life Sciences, Inc.). The specific SERT-mediated $^{[3H]}$5-HT was determined by subtracting the non-specific uptake. The remaining cell lysate was used to determine protein concentration by the Bradford method (Beyotime Institute of Biotechnology), with BSA as a standard.

Western blot analysis for GLP-1R and SERT. IEC-6 cells were washed with ice-cold PBS three times and lysed in 100 μl RIPA lysis buffer (Beyotime Institute of Biotechnology) with a protease inhibitor cocktail. Following centrifugation at 12,000 x g for 10 min at 4˚C, supernatants were collected and transferred to 1.5 ml centrifuge tubes. The protein concentration was determined using a BCA assay (26) according to the manufacturer's protocols (Thermo Fisher Scientific, Inc.). Total proteins were dissolved in lithium dodecyl sulfate sample buffer (0.5% lithium dodecyl sulfate; 62.5 mM Tris·HCl; 2.5% glycerol; 0.125 mM EDTA; pH 8.5). Samples (30 μg/lane) were separated by 10% (w/v) SDS-PAGE. After being transferred to PVDF membrane, washed and blocked with 5% non-fat dry milk in 0.1% Tween/Tris-Buffered Saline (Beyotime Institute of Biotechnology) for 1 h at room temperature, the membranes were incubated with goat polyclonal anti-GLP-1R primary antibody (1:500; cat. no. sc-34637; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-SERT primary antibody (1:500; cat. no. AB9726; EMD Millipore) and mouse monoclonal anti-GAPDH primary antibody (1:5,000; cat. no. M0002; CMC Scientific) overnight at 4˚C. Membranes were then washed three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:3,000; cat. no. BS13278; Bioworld Technology, Inc.), goat anti-mouse (1:3,000; cat. no. BS12478; Bioworld Technology, Inc.) or rabbit anti-goat (1:3,000; cat. no. BS10008; Bioworld Technology, Inc.) secondary antibodies for 60 min at 37˚C. Protein bands were visualized using the ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and images captured using a Bio-Rad Gel Imaging system (Bio-Rad Laboratories, Inc.). Protein expressions were normalized to GAPDH and were analyzed using Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR for the detection of SERT mRNA. Total RNA was extracted from the cells using TRIzol® reagent (Shanghai Pufei Biotechnology Co., Ltd.), according to the manufacturer's protocol, under RNase-free conditions. RNA concentrations were determined using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized from total RNA (500 ng/each sample) using the PrimeScript RT Master mix

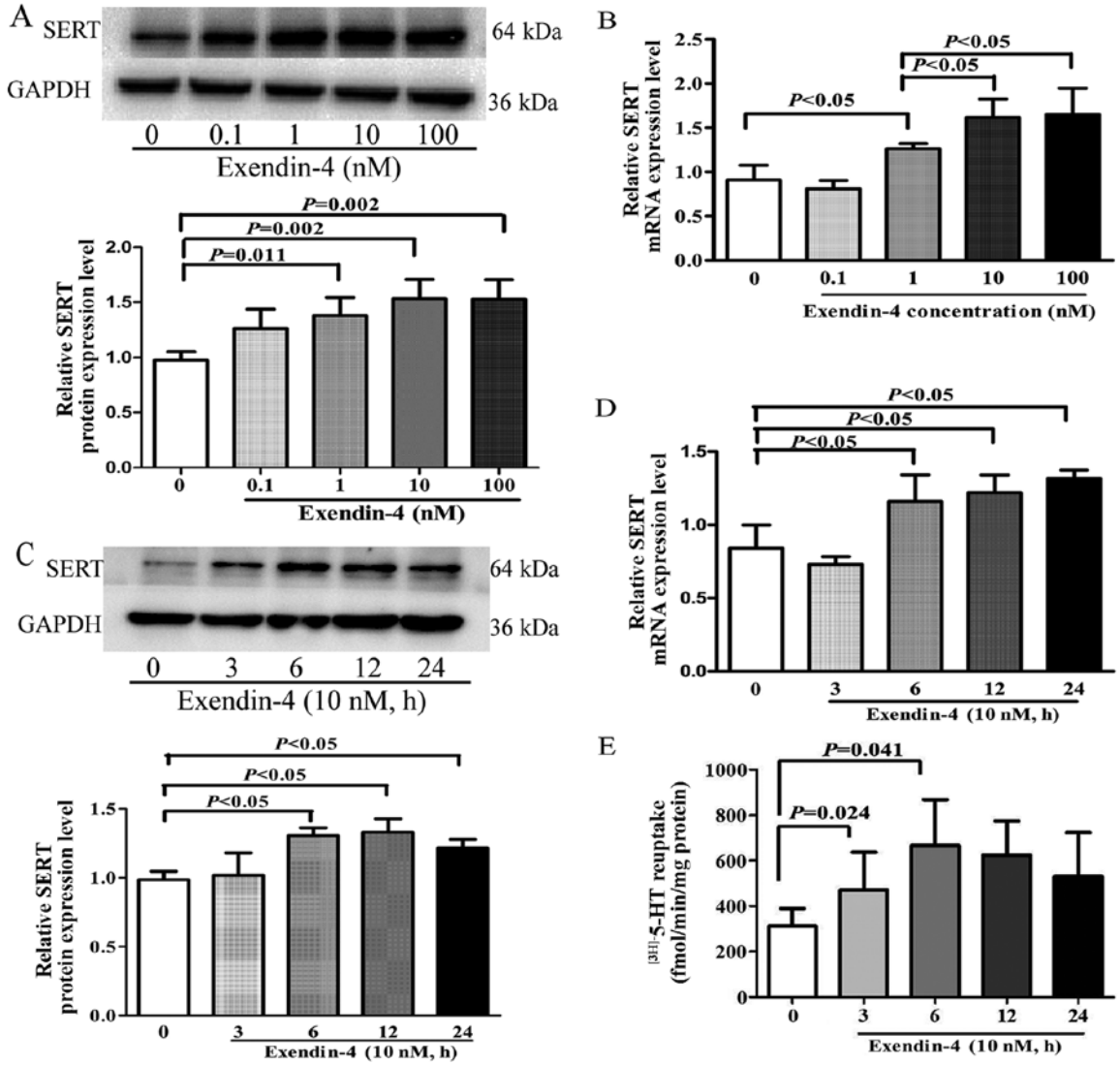

Figure 1. Effects of exendin-4 on SERT function and expression in IEC-6 cells. (A and B) IEC-6 rat intestinal epithelial cells were treated with various concentrations of exendin-4 (0, 0.1, 1, 10 and 100 nM) for 12 h and the (A) protein and (B) mRNA expression levels of SERT were determined by western blotting and RT-qPCR analysis, respectively. (C-E) IEC-6 cells were treated with exendin-4 (10 nM) for 0, 3, 6, 12 and 24 h and the (C) protein and (D) mRNA expression levels of SERT were determined by western blotting and RT-qPCR analysis, respectively. (E) 5-HT reuptake in IEC-6 cells measured. GAPDH was used as a loading control for western blotting; β-actin was used as an internal control for RT-qPCR. Values are presented as the mean ± SD of 3 independent experiments. 5-HT, 5-hydroxytryptamine (serotonin); RT-qPCR, reverse transcription-quantitative PCR; SERT, serotonin-selective reuptake transporter.

(Takara Biotechnology Co., Ltd.). The reverse transcription conditions are as follows: 37˚C for 15 min and 85˚C for 5 sec. cDNA (2 μl) was amplified by PCR using the following primers: SERT, forward 3'-GACTCCTCCCCTCTAAGCCA-5', reverse 3'-CACGGAAAGAAGTGGTCGGA-5'; β-actin, forward 3'-CTAAGGCCAACCGTGAAAAG-5', reverse 3'-TCTCAG CTGTGGTGGTGAAG-5'. qPCR was performed using a 20 μl reaction volume and SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) with an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the following thermocycling conditions: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. The quantitation cycle (Cq) values of all genes were obtained; the expression values of investigated genes were normalized to β-actin mRNA levels, and the relative expression was determined using the $2^{-\Delta\Delta Cq}$ method (27). All reactions were performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS Statistics 18.0.0 software (SPSS, Inc.). Data are expressed as the mean ± SD. For multiple comparisons, a parametric one-way ANOVA, two-way ANOVA and Bonferroni's post-hoc test were used. GraphPad Prism software 5.0 (Prism; GraphPad Software, Inc.) was used for plotting figures. P<0.05 was considered to indicate a statistically significant result.

Results

Effects of exendin-4 on SERT expression and 5-HT reuptake in IEC-6 cells. In a previous *in vivo* study, the GLP-1 analogue exendin-4 was revealed to upregulate SERT expression and decrease 5-HT levels in the intestinal mucosa of colonic sensitized rats (10). However, the mechanisms governing this are yet to be determined. The present study aimed to investigate whether exendin-4 modulated SERT expression and to

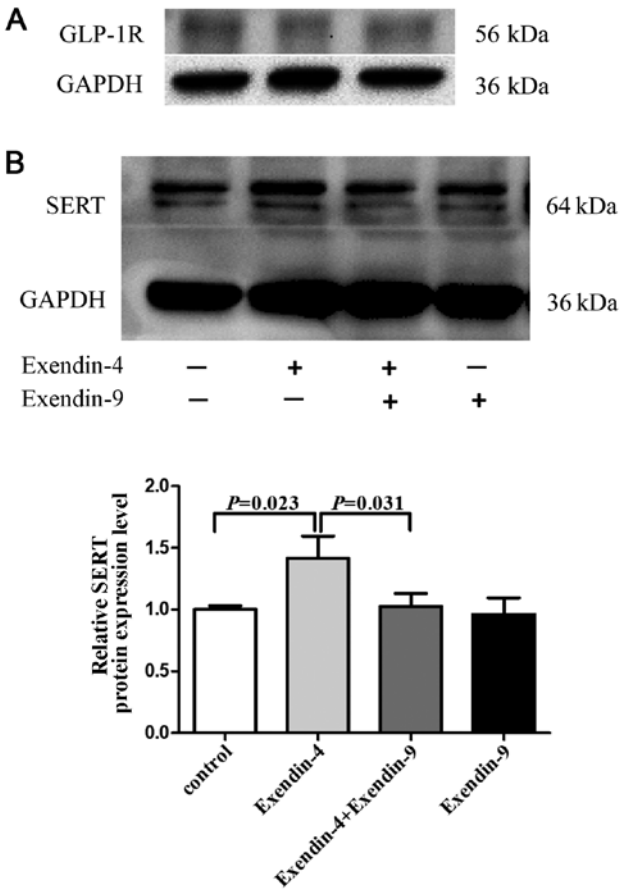

Figure 2. GLP-1R expression and its role in the effect of exendin-4 on SERT protein expression in IEC-6 rat intestinal epithelial cells. (A) GLP-1R protein expression was detected using western blot analysis. (B) IEC-6 cells pre-treated with GLP-1R antagonist exendin-9 (10 μM) for 1 h prior to the experiment, and with exendin-4 (10 nM) with or without exendin-9 (10 μM) co-treatment for a subsequent 12 h, and the effects of GLP-1R on SERT protein expression were determined by western blotting. GAPDH was used as an internal control. GLP-1R, glucagon-like peptide-1 receptor; SERT, serotonin-selective reuptake transporter.

identify the intracellular signaling mechanisms behind this *in vitro*. IEC-6 cells were treated for 12 h with exendin-4 at doses of 0, 0.1, 1, 10 and 100 nM; the results demonstrated that SERT protein and mRNA expression levels were significantly increased compared with the untreated cells (Fig. 1A and B, respectively), and expression was highest when doses were 10 and 100 nM. To determine whether exendin-4 regulated SERT expression in a time-dependent manner, IEC-6 cells were treated with 10 nM exendin-4 for 0, 3, 6, 12 and 24 h. The results demonstrated that the expression levels of SERT protein and mRNA were significantly increased following exendin-4 treatment for 6, 12 and 24 h (Fig. 1C and D, respectively). Additionally, to observe the effect of exendin-4 on SERT activity, a kinetic study was performed to identify 5-HT reuptake rates; the results revealed that 5-HT reuptake as significantly enhanced at 3 and 6 h in IEC-6 cells treated with 10 nM exendin-4 (Fig. 1E).

Exendin-4 effects SERT protein expression through GLP-1R. To examine whether exendin-4 upregulated SERT expression by binding to GLP-1R, the GLP-1R antagonist exendin-9 was used to pre-treat IEC-6 cells. GLP-1R was expressed in IEC-6 cells (Fig. 2A). SERT protein expression in IEC-6 cells significantly increased after exendin-4 treatment, and this effect was blocked by pretreatment with the GLP-1R antagonist exendin-9 (Fig. 2B). These results suggested that SERT stimulation was partially affected by GLP-1R-mediated signaling, indicating that the upregulatory effect of exendin-4 on SERT expression was mediated through GLP-1R.

AC/PKA signaling pathway serves a role in the upregulation of SERT expression by exendin-4. To determine whether exendin-4 influenced SERT expression via the AC/PKA signaling pathway, IEC-6 cells were treated with exendin-4 (10 nM) in the presence or absence of the AC activator forskolin or the AC inhibitor SQ22536. PKA activity was significantly upregulated in cells treated with exendin-4. and the effect was reversed by pretreatment with AC inhibitor SQ22536 for 30 min (Fig. 3A). Treatment with forskolin or exendin-4 significantly increased SERT protein expression in IEC-6 cells (Fig. 3B). The results indicated that the exendin-4induced SERT expression was also inhibited by pretreatment with SQ22536. These results demonstrated that SQ22356 inhibited exendin-4-induced SERT expression (Fig. 3B). Treatment with PKA inhibitor H89 (10 μM) also exhibited a similar blocking effect in IEC-6 cells (Fig. 3C).

Discussion

In our previous study, the GLP-1 analogue exendin-4 was found to reduce visceral hypersensitivity and increase SERT protein expression in colonic sensitized rats (10). As previously reported, 5-HT is associated with the visceral sensitivity of the gastrointestinal tract (28,29). Therefore, decreased SERT expression may led to a decreased capacity to remove 5-HT from the interstitial space.

IBS animal models and patient studies have previously revealed decreased SERT expression in the colon, suggesting that abnormal serotonergic signaling may contribute to visceral hypersensitivity during IBS (17,30). In a previous study, decreased SERT protein expression was observed in colonic sensitized rats, and after exendin-4 treatment, SERT protein expression increased and 5-HT levels decreased in colonic sensitized rats (10). The present study demonstrated that in IEC-6 cells, exendin-4 upregulated SERT protein expression and mRNA level *in vitro*. The 5-HT reuptake rate was significantly enhanced, but the expression of SERT mRNA and protein was not significantly increased in IEC-6 cells 3 h after treatment with 10 nM exendin-4. This observed effect may be correlated to elevated SERT activity. Considering SERT activity in intestinal epithelial cells, this regulation may be an effect on the allosteric site of the membrane protein (29). Additionally, the results of the current study revealed that SERT mRNA and protein expression increased following exendin-4 treatment at 6, 12 and 24 h, and SERT expression reached a peak at 6 h following treatment. The 5-HT re-uptake rate was also significantly increased at 6 h following treatment with 10 nM exendin-4. These results, which were in accordance with those from the studies of Gill *et al* (31) and Kerckhoffs *et al* (16), suggested that the augmented expression of SERT led to an elevated capacity to remove 5-HT from the interstitial space.

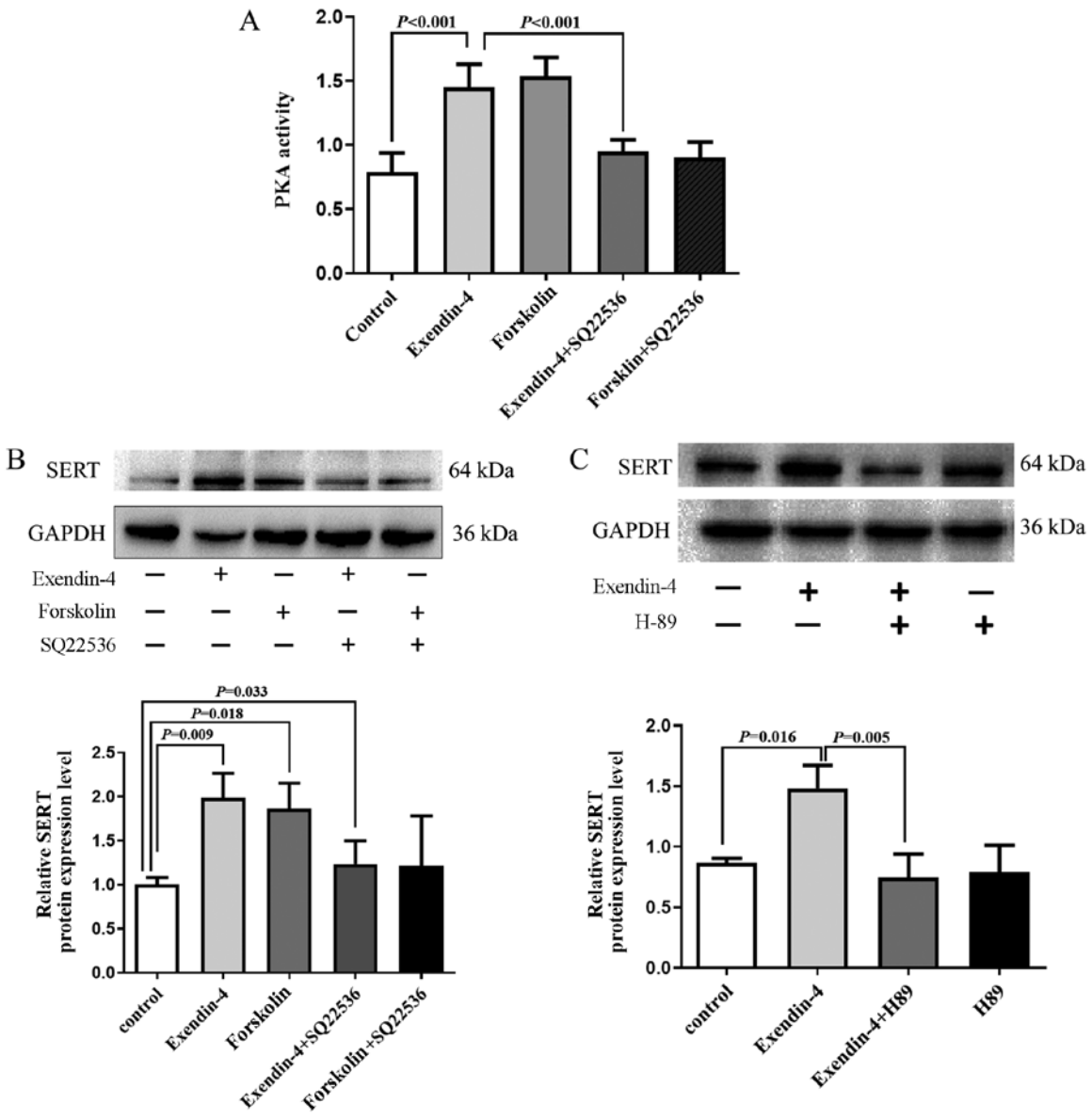

Figure 3. Adenosine cyclophosphate/PKA cell signaling pathway in exendin-4-induced SERT protein expression *in vitro*. IEC-6 rat intestinal epithelial cells were pretreated with SQ22536 (10 μM) or H89 (10 μM) for 30 min in the presence of exendin-4 (10 nM), or individually treated with exendin-4 (10 nM) or forskolin (5 μM) for an additional 12 h. (A) PKA activity was detected using a non-radioactive PKA kinase assay kit (Type I). (B and C) SERT protein expression was examined by western blot analysis; GAPDH was used as an internal control. Data are expressed as the mean ± SD from three independent experiments. PKA, protein kinase A; SERT, serotonin-selective reuptake transporter.

To investigate whether exendin-4 regulated SERT protein expression through GLP-1R, the GLP-1R specific antagonist exendin-9 was used. The results indicated that exendin-9 reversed the upregulation of SERT protein expression induced by exendin-4, and this suggested that exendin-4 upregulated SERT protein expression through GLP-1R. It has previously been shown that GLP-1R activation can stimulate cAMP formation and subsequently induce PKA activity (13). SERT expression can be regulated by a variety of stimuli, including cAMP, hormones and epidermal growth factor (14,15,32,33). Therefore, the AC/PKA signaling pathway was investigated to determine whether exendin-4 could trigger intracellular signaling. The present results indicated that the AC/PKA signaling pathway mediated SERT expression induced by exendin-4.

There are a number of limitations of the present study. As this was an *in vitro* study involving a single cell line, further investigations using different cell lines is required. Additionally further studies into the effect of extendin-4 on 5-HT in animal models is required. Another limitation of this study is that this study did not focus on specific subtypes of IBS. As the majority of patients with IBS suffer from abdominal pain that is thought is to be caused by visceral sensitivity, this study was based on the assumption that extendin-4 and its influence on SERT expression and function is a common characteristic of all types of IBS. However, further investigation is needed to verify this.

In conclusion, exendin-4 may modulate SERT expression and 5-HT reuptake in intestinal epithelial cells. Combined with the results of previous studies, the GLP-1R/AC/PKA/SERT signaling pathway may be the signaling pathway of exendin-4 that is used to reduce visceral hypersensitivity. However, the mechanism by which exendin-4 alleviates visceral sensitivity in rat IBS models requires further study.

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Availability of data and materials

The data used and analyzed in this study are available from the corresponding author on reasonable request.

Authors' contributions

XC participated in the study design, data acquisition and analysis, and drafting, interpretation and writing of the manuscript. XZhao, YW and YY participated in the statistical analysis. HZhang participated in the study concept and design, study supervision, interpretation of data, and critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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