International Journal of Pharmaceutics 368 (2009) 31–36

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Effect of adrenergic stimulation on drug absorption *via* passive diffusion in Caco-2 cells

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ARTICLE INFO

Article history:
Received 5 June 2008
Received in revised form 4 September 2008
Accepted 28 September 2008
Available online 9 October 2008

Keywords:
Enteric nervous system
Adrenaline
Clonidine
Adrenoceptor
Passive diffusion
Paracellular transport

ABSTRACT

It is well known that the enteric nervous system (ENS) regulates the movement and function of the small intestine, but the effects of ENS on drug absorption from the small intestine still remain to be clarified. Focusing on adrenergic effect, we tried to evaluate how adrenergic stimulation influences the drug absorption *via* passive diffusion using Caco-2 cells as model epithelial cells, a terminal effector of ENS. Adrenaline, an adrenergic agonist, did not affect the transport of small molecules such as antipyrine, phenacetin and mannitol, but decreased the transport of large molecules such as FITC-dextran (FD)-20 and FD-40 without transepithelial electrical resistance (TEER) change. These results suggested that the transport of large molecules *via* paracellular route would be attenuated by adrenergic stimulation. Only clonidine, an α_2-agonist, among selective adrenoceptor agonists decreased FD-40 transport across Caco-2 cell monolayers and the agonist also decreased intracellular cAMP. Furthermore, H-89, a protein kinase A inhibitor, significantly decreased FD-40 transport and dibutyryl cAMP, a cAMP derivative, increased it. These results suggest that the decrease in FD-40 transport would be mainly attributed to the decrease in intracellular cAMP and subsequent decrease in PKA activity *via* α_2-receptor stimulation.

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1. Introduction

The small intestine is the organ where most of the nutrients from ingestion are absorbed, and plays an essential role in maintenance of homeostasis. Since the physicochemical properties such as volume, pH, osmolarity and viscosity of luminal contents are always variable, the small intestine has to adapt its own function to such luminal environments to maintain efficient absorption of nutrients. This adaptation is controlled by the enteric nervous system (ENS), which is an intrinsic nervous system extending throughout the length of the gastrointestinal tract.

ENS is recognized as an independent integrative system with structural and functional properties similar to those of the central nervous system, and more than 20 neurotransmitters participate in the neural network (Furness, 2000). They include acetylcholine, monoamines, peptides, amino acids, purines and gasses such as NO (Hansen, 2003). ENS consists of two ganglionated plexuses, the myenteric and submucosal plexuses. Myenteric ganglia are distributed between the outer longitudinal and circular muscle layers, and myenteric neurons mainly regulate contractile activity such as segmentation, mixing movements and peristalsis (Kunze and Furness, 1999; Furness, 2000). On the other hand, submucosal ganglia are embedded in the submucosa, and submucosal neurons are mainly involved with epithelial function and blood flow (Hildebrand and Brown, 1990; Cooke and Reddix, 1994). From the aspect of type of neurons, ENS is composed of sensory, interconnecting and motor neurons. Sensory neurons, expressing sensory receptors on the cell membrane, convert external stimuli to electrical impulse, and release neurotransmitters toward interconnecting neurons. Interconnecting neurons communicate only to other neurons, and provide connection between sensory and motor neurons. Motor neurons project their processes to the smooth muscle or the epithelial cells, and directly regulate their function through release of neurotransmitters.

The effect of ENS on the functions of small intestine has been intensively studied with respect to the regulation of the smooth muscle (Liu and Coupar, 1996; Kunze and Furness, 1999; Furness, 2000; Bayer et al., 2003) or the transport of water and/or electrolytes (Cooke, 1989; Hildebrand and Brown, 1990; Cooke and Reddix, 1994). However, the information about the effect of ENS on drug absorption from small intestine is still very limited (Hayden and Carey, 2000; Neunlist et al., 2003; Hiraoka et al., 2005). The intercellular junctional complexes that are formed between enterocytes, restrict the drug absorption *via* paracellular diffusion (Miyoshi and Takai, 2005). As to the transcellular transport, on the other hand, lipid bilayer and various transporters expressing

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0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.09.050

on the membrane participate in the drug absorption *via* passive diffusion and active uptake/efflux, respectively (Ito et al., 2005). These functions of epithelial cells are important determinants for drug absorption from the small intestine, but the mechanisms how the neural stimulation affects the functions remain to be clarified. Therefore, it is important to elucidate the mechanisms for understanding the change in drug absorption under various physiological conditions. Particularly, it has recently been found that the abnormal activities of ENS were associated with some serious intestinal diseases such as inflammatory bowel disease and irritable bowel syndrome (Atkinson et al., 2006; Villanacci et al., 2008). The abnormalities would affect the intestinal permeability and motility, but the details and mechanisms have not been well characterized.

Our previous reports have demonstrated that chronic depletion of serotonin, which is one of the most important neurotransmitters and profoundly related in the regulation of intestinal function (McLean and Coupar, 1998), significantly enhanced the transport activity of P-glycoprotein on the brush border membrane of epithelial cells (Hiraoka et al., 2005). In relation to passive diffusion, we previously reported that the absorption of phenol red, a poorly absorbable compound, is suppressed by the stimulation of adrenergic neuron in the vascular-luminal perfusion study or the *in vitro* transport study using rat small-intestinal sheet (Higaki et al., 2004). Although phenol red was employed as a marker of paracellular transport then, it has been recently suggested that phenol red is secreted by an organic anion efflux system (Itagaki et al., 2005, 2008), which means that the previous results (Higaki et al., 2004) may include the change in the activity of efflux system. Some adrenergic nerves directly penetrate into the intestinal mucosa (Furness and Costa, 1980; Cooke, 1988; Sarna and Otterson, 1989), and noradrenaline is well known to be one of important neurotransmitters in ENS (Cooke, 1994). Furthermore, it has been reported that catecholamines at least partially promote the antisecretory action *via* α_2-receptors located on epithelial cells (Valet et al., 1993). As described above, however, the effect of adrenergic stimulation on the drug transport *via* passive diffusion still remains to be clarified. Therefore, we revaluated the effect of ENS on passive diffusion in the present study with antipyrine and phenacetin as markers of transcellular transport, mannitol and FITC-dextrans as markers of paracellular transport by using Caco-2 cells, a model of intestinal epithelial cells, a terminal effector of ENS.

2. Materials and methods

2.1. Materials

Antipyrine, phenacetin, FITC-dextrans with molecular weights of 4000 (FD-4), 20,000 (FD-20) and 40,000 (FD-40), adrenaline, phenylephrine, clonidine, dobutamine, metaproterenol, dibutyryl cAMP (DBcAMP) and H-89 were purchased from Sigma–Aldrich (St. Louis, MO, USA). [^{3}H]Mannitol was purchased from PerkinElmer Inc. (Waltham, MA, USA). All other reagents were analytical grade commercial products.

2.2. Cell culture

Caco-2 cells, obtained from the cell bank of the Intestine of Physical and Chemical Research (Ibaragi, Japan), were grown in a CO_2 incubator (MCO-175, Sanyo, Tokyo, Japan) maintained at 37 °C, 5% CO_2 and 90% relative humidity, using Dulbecco's Modified Eagle's medium (Sigma–Aldrich) containing 10% fetal bovine serum, 20 μg/mL gentamycin, and 100 U/100 μg/mL penicillin/streptomycin (Sigma–Aldrich). The culture medium was changed every other day. Caco-2 cells were seeded at 1×10^5 on a Transwell (growth area, 1.12 cm^2; Corning Inc., Corning, NY, USA). The cells were grown for 14–20 days for study.

2.3. Transport study

After preincubation with Ringer's solution (pH 7.4) containing 1.2 mM NaH_2PO_4, 125 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 10 mM $NaHCO_3$ and 2 mg/mL D-glucose for 25 min, Ringer's solution containing each passive diffusion marker was placed in the apical side (0.5 mL) of Caco-2 cell monolayers on a Transwell. Adrenergic agonists or intracellular signal modulators were added into the basal side (1.5 mL) at the same time. Samples (0.2 mL for antipyrine, phenacetin and mannitol; 0.5 mL for FD-4; 0.75 mL for FD-20 and FD-40) were drawn out of the basal side at 10-min intervals to 90 min. An equal volume of Ringer's solution containing each adjuvant was immediately added to the basal side after each sampling. Transepithelial electrical resistance (TEER) was simultaneously determined using a Millicell-ERS resistance system (Millipore, Billerica, MA, USA).

2.4. Measurement of intracellular cAMP level

After preincubation with Ringer's solution (pH 7.4), adrenaline or clonidine was added into the basal side of Caco-2 cell monolayers on a Transwell. After incubation for 2, 6, 10, 20, 45 or 90 min, the reaction was stopped by adding 35 μL of 1N HCl solution. After 20 min, the suspension was centrifuged for 10 min at $1000 \times g$. The cAMP levels of the supernatant were determined by enzyme immunoassay using cyclic AMP EIA Kit (Cayman Chemical, Ann Arbor, MI, USA). Samples, the cAMP antibody and the tracer (acetylcholinesterase linked to cAMP) were incubated at 4 °C for 18 h in 96-well microplate pre-coated with mouse monoclonal antibody. After washing wells, quantification of the tracer was achieved by measuring its enzyme activity with Ellman's reagent, where the absorbance of final product, 5-thio-2-nitrobenzoic acid, was determined at 410 nm using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). Protein amount of samples was determined by DC Protein Assay Kit (Bio-Rad Laboratories).

2.5. Analytical method

FITC-dextrans were determined fluorospectrophotometrically at 485 nm for excitation and at 515 nm for emission (F4500 fluorescence spectrophotometer, Hitachi, Tokyo). Radioactivity of [^{3}H]mannitol was determined in a liquid scintillation counter (LS-232, Beckman, Fullerton, CA, USA) after the scintillation medium (Clear-Sol II, Nacalai Tesque, Kyoto, Japan) was added to each sample.

Antipyrine and phenacetin were determined by HPLC, which consists of a model LC-6A HPLC pump (Shimadzu, Kyoto), a model SPD-6A system controller (Shimadzu) set at 254 nm for antipyrine, or set at 243 nm for phenacetin. A Synergi Fusion-RP column (150 mm × 4.6 mm i.d.; particle size, 4 μm; Phenomenex Inc., Torrance, CA, USA) was used at room temperature. The mobile phase for antipyrine was 20 mM phosphate buffer (pH 7.4):methanol (65:35, v/v) delivered at 1.0 mL/min, and that for phenacetin was 0.025% phosphoric acid:methanol (40:60, v/v) delivered at 1.0 mL/min. The concentration range of standard curves was 0.25–50 or 0.1–50 μM for antipyrine or phenacetin, respectively. The squared correlation coefficient for standard curves was over 0.999. The coefficient of variation (CV) ranged from 0.36 to 4.6%.

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