Absorption-Enhancing Effect of Nitric Oxide on the Absorption of Hydrophobic Drugs in Rat Duodenum

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Received 17 July 2015; revised 4 September 2015; accepted 16 September 2015

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24677

ABSTRACT: Nitric oxide (NO), an endogenous gas that plays a versatile role in the physiological system, has the ability to increase the intestinal absorption of water-soluble compounds through the paracellular route. However, it remains unclear whether NO can enhance the absorption of hydrophobic drugs through the transcellular route. In this study, we examined the absorption-enhancing effect of NO on intestinal permeability of hydrophobic drugs in rat intestine. The pretreatment of rat gastrointestinal sacs with NOC7, a NO-releasing reagent, significantly increased the permeation of griseofulvin from mucosa to serosa in the sacs prepared from the duodenum, but not in those prepared from the other regions such as jejunum, ileum, and colon. The absorption-enhancing effect of NOC7 on the duodenal permeation varied depending on the hydrophobicity of the drugs used. Furthermore, NOC7 treatment was found to be apparently ineffective on the griseofulvin permeation in the duodenum pretreated with dithiothreitol (DTT) that was used as a mucus remover, even though the permeation was increased by pretreatment with DTT alone. These results suggest that NO increases the absorption of hydrophobic drugs through the transcellular route in the duodenum by modulating the mucus layer function.

Keywords: nitric oxide; absorption enhancer; transcellular route; mucus layer; passive diffusion; permeability; duodenum; log P

INTRODUCTION

Drug absorption across the intestinal epithelium is mediated by two distinct mechanisms: the transcellular and paracellular routes.[1] The former is mostly involved in the absorption of hydrophobic compounds, depending on passive diffusion, whereas the latter is involved in the absorption of highly water-soluble compounds, which can pass through the lateral intercellular space between intestinal epithelial cells. Because many drugs are generally hydrophobic, the transcellular route is a major pathway for their absorption in the gastrointestinal tract. Thus, approaches for optimization of drug absorption via the transcellular route are of pharmaceutical importance for improving bioavailability. Numerous efforts to improve poorly absorbable drugs have been made; however, most of them were intended to modulate the physicochemical properties of drugs by solubilization or nanosizing.[2–4] Therefore, absorption enhancers targeting the transcellular route are yet to be developed.

On the other hand, various absorption enhancers targeting the paracellular route, such as surfactants, bile salts, chelating reagents, and fatty acids have been investigated to enhance the permeability of poorly absorbable hydrophilic drugs, which include small peptides.[5,6] More recently, it has been reported that nitric oxide (NO), an endogenous gas that plays a versatile role in the physiological system,[7] has the ability to increase the permeability of water-soluble compounds through paracellular route.[8,9] The absorption-enhancing action of NO has been observed in Caco-2 cell monolayers in a reversible manner without any loss of cell viability or leakages of intracellular enzymes.[10] In addition, several groups have independently demonstrated that the intestinal absorption of fluorescein isothiocyanate-dextran 4000 (FD-4) or 5(6)-carboxyfluorescein, which are paracellular markers, was remarkably enhanced in the presence of NO donors *in vitro* and *in situ* absorption studies.[11–14] The mechanisms underlying the absorption-enhancing effect involve dilatation of the tight junction in the intestinal epithelium, which facilitate absorption via the paracellular route.[12] Therefore, it is suggested that NO donors could be excellent absorption enhancers for improving the intestinal absorption of poorly absorbable drugs through paracellular route. However, studies on the effect of NO on the transcellular route are scarce.

In this study, we examined if NO could alter the intestinal permeability of hydrophobic drugs in rat intestine using 1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) as a NO-releasing reagent. In order to evaluate the effect of NO on the passive transcellular transport, we chose griseofulvin as a probe with the properties of high permeability and high hydrophobicity from the compounds categorized into the biopharmaceutics classification system Class II.[15]

MATERIALS AND METHODS

Materials

NOC7 was obtained from Dojindo Laboratories (Kumamoto, Japan). Griseofulvin, diclofenac, theophylline, and dithiothreitol (DTT) were obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Antipyrine and FD-4 (MW 4000) were obtained from Sigma–Aldrich Company Ltd. (St. Louis, Missouri). All other reagents were of analytical grade.

Abbreviations used: DTT, dithiothreitol; FD-4, fluorescein isothiocyanate-dextran 4000; KHB, Krebs-Henseleit bicarbonate; NO, nitric oxide; NOC7, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene; P_{app}, apparent permeability coefficient; UWL, unstirred water layer.

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Journal of Pharmaceutical Sciences

Animals

Male Wistar rats (8 weeks old) were obtained from the Tokyo Laboratory Animals Science Company, Ltd. (Tokyo, Japan). The animals were housed in a clean room maintained at 23 ± 2°C with a relative humidity of 55 ± 10% and a 12 h light–dark cycle. They were allowed free access to food and water. The animal experimental protocols were approved by the Tokyo University of Pharmacy and Life Sciences Committee on the Care and Use of Laboratory Animals.

Drug Permeation Study Using the *In Vitro* Sac Method

To evaluate intestinal drug permeability, experiments were conducted using tissue sacs prepared from rat small intestine, as previously described.[16] Rats fasted overnight for 18 h were anesthetized using Somnopentyl® (pentobarbital sodium, 50 mg/kg, i.p.), the abdomen was opened by making a midline longitudinal incision, and intestinal segments were removed from the duodenum, jejunum (5 cm below the Treitz ligament), ileum (5 cm above the cecum), and colon (5 cm below the cecum) in lengths of 4, 6, 6, and 4 cm, respectively. The isolated intestinal segments were carefully washed with saline and cannulated at both ends using glass tubing attached to a plastic syringe, which was used to inject and replace the solution in the lumen (mucosal solution, 5 mL). The segments were filled with Krebs-Henseleit bicarbonate (KHB) buffer (pH 6.5), and then immersed in 40 mL of KHB buffer (serosal solution) and preincubated for 10 min. The mucosal solution was replaced with 5 mM NOC7 and incubated for 30 min. Subsequently, the mucosal side was washed using KHB buffer, and the drug permeation study was started by injecting 5 mL of KHB buffer containing each test compound (200 μM) into the mucosal side space. To examine the effect of mucus layers on drug permeation, the mucosal side was pretreated with 10 mM DTT for 10 min before NOC7 treatment initiation. Sampling of the serosal solution was performed at 0, 10, 20, 30, 40, 60, 90, and 120 min after administration. At the end of the experiment, the surface area of each intestinal segment was calculated by measuring the length and width with a ruler. The mucosal and serosal solutions, and KHB buffer were maintained at 37°C. The apparent permeability coefficients (P_{app}) were calculated according to the following equation: P_{app} (cm/s) = $dQ/dt \cdot 1/C_0S$, where dQ/dt is the appearance rate of the test compound in the serosal solution, C_0 is the initial concentration of the test compound in the mucosal solution, and S is the surface area of intestinal lumen.

Drug Absorption Study using the *In Situ* Closed-Loop Method

Absorption experiments were performed by an *in situ* closed-loop method, as described previously with some modifications.[17] The rats were fasted overnight for 18 h and anesthetized using Somnopentyl®. The duodenum was exposed by a midline abdominal incision, and a closed loop was prepared using a segment of the duodenum. The duodenum lumen was washed with prewarmed saline, and the remaining was expelled using air. The proximal and distal ends of the duodenum were cannulated using glass tubing connected to the three-way stopcocks. For the experiments involving NOC7 pretreatment, the duodenum loop was treated for 30 min with KHB buffer containing 5 mM NOC7, which was introduced through the three-way stopcocks. Subsequently, the lumen of the duodenal segment was washed with KHB buffer. A test solution (2.5 mL) containing griseofulvin (20 μM) and FD-4 (250 μM), kept at 37°C, was introduced into the loop. Whole blood (0.2 mL) was collected from the portal vein at the indicated time, and plasma samples were obtained from the whole blood by centrifugation.

Analytical Methods

The concentration of the test compounds in the serosal solution and plasma sample were measured using reversed-phase high performance liquid chromatography system (LC-2000Plus; JASCO Company, Tokyo, Japan) equipped with a variable wavelength UV-visible detector (UV-2070plus; JASCO Company) and a fluorescence detector (FP-2025plus; JASCO Company). Griseofulvin was separated on an octadecylsilyl (ODS) column (COSMOSIL 5C18-AR-II column, 150 × 4.6 mm^2) using a mobile phase consisting water and acetonitrile (3:2, v/v) at a flow rate of 1.0 mL/min at 30°C and monitored fluorometrically at excitation/emission wavelengths of 492/515 nm. Diclofenac was separated on the ODS column using a mobile phase consisting methanol and water (3:1, v/v) containing 0.1% phosphoric acid at a flow rate of 1.0 mL/min at 40°C, and monitored spectrophotometrically at a wavelength of 286 nm. Similarly, antipyrine was separated on the ODS column using a mobile phase (methanol/50 mM potassium dihydrogen phosphate = 1:1, v/v) and monitored at 254 nm. Theophylline was separated on the ODS column using a mobile phase (methanol/water = 1:4, v/v) containing 1% acetic acid and monitored at 270 nm. The concentrations of FD-4 were determined using a fluorescence microplate reader (Varioskan™ Flash 2.4; Thermo Fisher Scientific Inc., Kanagawa, Japan) at excitation/emission wavelengths of 492/515 nm.

Statistical Analysis

All results are expressed as the mean ± SD. Statistical significance between groups was analyzed using the Student's *t*-test or ANOVA followed by Dunnett's method, and $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Regional Differences in the Effect of NO on the Intestinal Absorption of Hydrophobic Drugs

We first examined the effect of NOC7 on the intestinal permeability of griseofulvin in rat gastrointestinal sacs, which were prepared from duodenum, jejunum, ileum, and colon (Fig. 1). Griseofulvin permeation from mucosa to serosa across rat gastrointestinal sacs was significantly increased in the duodenum pretreated with NOC7 compared with the control condition (Fig. 1a). However, this enhancing effect was not observed in the sacs prepared from the jejunum, ileum, and colon, which indicated a regional specificity of the NO effect (Figs. 1b–1d). It is notable that the absorption-enhancing effect of NO on the absorption of FD-4, a paracellular marker, has been reportedly observed in the entire intestinal region, with the greatest enhancement observed in the colon.[11,13] This discrepancy between the present and the previous results may be attributed to the differences in the mechanism of NO action between the transcellular and paracellular routes. Therefore, in subsequent experiments, we examined the effect of NOC7 on drug permeation in the duodenum by the transcellular route.

 DOI 10.1002/jps.24677