Nitric Oxide Donors Enhance Rectal Absorption of Macromolecules in Rabbits

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Purpose. The objective of this investigation is to evaluate the potential of nitric oxide (NO) donors as a new class of absorption enhancers which may act on intestinal epithelial cells through epithelial actions of the chemical mediator, NO.

Methods. Suppositories containing NO donors and insulin were administered into the rabbit rectum. After administration of the suppository, blood samples were collected from the auricular vein. The plasma insulin and glucose concentrations were determined.

Results. The NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP, 4 mg) induced a significant increase in the rate of insulin absorption from the rectum. Administration of a suppository containing SNAP without insulin affected neither the plasma insulin nor the plasma glucose concentration. Other NO donors, NOR1 and NOR4, also induced increases in the insulin absorption. The absorption enhancement effect of SNAP was inhibited by coadministration of the NO scavenger carboxy-PTIO. SNAP also enhanced FITC-dextran (MW 4,000) absorption. Little cytotoxicity of SNAP (3.0 mg/ml) as assessed in terms of the rate of lactate dehydrogenase (LDH) release from Caco-2 cells was detected for 2 h of incubation.

Conclusions. These findings suggest that NO enhanced macromolecular absorption from the rectum without mucosal cell damage, and that NO donors can act as potent absorption enhancers.

KEY WORDS: rectal mucosa; absorption enhancer; nitric oxide.

INTRODUCTION

The paracellular transport route is the most important route across intestinal epithelial cell layers for part of hydrophilic compounds or macromolecules such as polypeptides. This pathway is restricted by the tight junctions at the apical side of mucosal epithelial cells. The advantages and limitations of coadministration of absorption enhancing agents (absorption enhancers) for increasing the intestinal absorption of poorly absorbed drugs have been exploited in many studies (1). The absorption enhancement effects of these enhancers are thought to be mediated by the induction of a change in fluidity of the mucosal cell membrane by the enhancers, interaction of the enhancers with cell membrane proteins, causing membrane perturbation (2), or chelation of calcium ions located between cells (3). However, a major concern regarding intestinal absorption enhancers is their potential to cause epithelial damage (4). For example, rectal epithelial and goblet cells are lost with many types of absorption enhancers such as sodium deoxycholate, sodium lauryl sulfate, ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA), and polyoxyethylene glycol (5,6). Other absorption enhancers such as polyoxyethylene ethers induce release of LDH and calcium from the colonic mucosa, indicating membrane damage (7). These observations suggest that in many cases, absorption enhancement might be associated with mucosal injury.

It is now well established that based on the results of studies involving cultured epithelial monolayers, intestinal epithelial permeability can be modulated by a number of factors, including extracellular pH (8), adenosine 3′,5′-cyclic monophosphate (9), insulin-like growth factors (10), activators of protein kinase C (11), and cytokines (12). Nitric oxide (NO) can regulate the permeability of Caco-2 monolayers which are widely used as models of intestinal epithelial monolayers (13). NO donors induce a dramatic increase in the permeability of Caco-2 monolayers with neither loss of cell viability nor LDH release (13). The increase in Caco-2 monolayer permeability induced by NO donors is reversible. Furthermore, dilation of the tight junctions between Caco-2 cells in the presence of NO donors can be detected by electron microscopy. These findings suggest that NO induces dilation of the tight junctions of the intestinal epithelial cells followed by an increase in the paracellular permeability as a physiological reaction with no cytotoxic effect on the mucosal epithelium.

The objective of the studies presented here was to evaluate the potential of NO donors as a new class of absorption enhancers which may act on intestinal epithelial cells through a chemical mediator, NO.

MATERIALS AND METHODS

Materials

S-Nitroso-N-acetyl-DL-penicillamine (SNAP), (±)-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide sodium salt (carboxy-PTIO) was purchased from Dojindo Lab. (Kumamoto, Japan). Bovine insulin (26 IU/mg), LDH-UV test-Wako, and Glucose test-Wako were obtained from Wako Pure Chemicals Ind. (Osaka, Japan). FITC-dextran (mean molecular weight 4,000; FD4) was purchased from Sigma Chemical Co. (St. Louis, MO). Heptakis(2,6-di-O-methyl)-β-cyclodextrin was donated by Nihon Shokuhin Kako Co. (Tokyo Japan). A suppository base, Witepsol H-15, was kindly supplied by Hüls Troisdorf (Troisdorf, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Seiyaku Co. (Tokyo, Japan). A stock solution of nonessential amino acids was obtained from Gibco-BRL (Grand Island, NY). Fetal calf serum was purchased from Biotech

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ABBREVIATIONS: C10, sodium caprate; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, sodium salt; DMEM, Dulbecco's modified Eagle's medium; DM-β-CyD, heptakis(2,6-di-O-methyl)-β-cyclodextrin; EDTA, ethylenediamine-N,N,N′,N′-tetraacetic acid; LDH, lactate dehydrogenase; NO, nitric oxide; SNAP, S-nitroso-N-acetyl-DL-penicillamine; FD4, FITC-dextran (molecular weight 4,000); NOR1, (±)-(E)-4-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide; NOR4, (±)-N-[(E)-4-ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexen-1-yl]3-pyridine carboxamide.

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International Ltd. (Australia). All other reagents used were of analytical grade.

Preparation of Suppositories

Hollow-type suppositories were prepared using Witepsol H-15 as previously described (14). Insulin and other reagents were dissolved at appropriate concentrations in an isotonic citrate buffer at pH 3.0. An aliquot of this solution was loaded into the cavity of the suppository. The opening at the hind part of the suppository was sealed with melted base material.

Animal Experiments

Male albino rabbits weighing 2.8–3.2 kg were given with free access to water. They were fasted for one night prior to each experiment. Each suppository was administered into the rectum as previously described (14). Both NO donors (SNAP, NOR1) except NOR4 or NO scavenger (carboxy-PTIO) were coadministerd with insulin or FD4. NOR4 was administered 2 h prior to the administration of insulin containing suppositories. After rectal administration of the suppository, 2 ml blood samples were collected at timed intervals from the auricular vein in a syringe containing EDTA. The blood was centrifuged at 3000 rpm for 15 min and the plasma was stored at -20°C until insulin and glucose assays were performed.

Determination of Insulin and Glucose in Plasma

The plasma insulin concentration was determined by enzyme immunoassay (EIA) with an EIA insulin Test-S kit (Medical & Biological Lab., Nagoya, Japan) based on the sandwich type immunoassay. The peak plasma insulin level (Cmax) and the peak concentration time (Tmax) were obtained from individual plasma insulin concentration-time curves. The area under the individual plasma insulin concentration-time curves from 0 to 6 h after rectal administration (AUC_{0-6}) was calculated using the trapezoidal rule.

The plasma glucose concentration was determined by a glucose test kit (Glucose test-Wako)-based *o*-toluidine-boric acid method (15). The decrease in the plasma glucose concentration (Δ Glc) was calculated from the area under the plasma glucose level versus time curve from 0 to 6 h after rectal administration of the suppository using the linear trapezoidal rule.

Determination of FD4 in Plasma

The plasma FD4 levels after rectal administration of a suppository containing FD4 (30 mg) was determined by fluorescence spectrophotometery (excitation 495 nm; emission 512 nm).

Cytotoxicity of NO Donors to Intestinal Epithelium

The levels of cytotoxicity of the NO donors or absorption enhancers were estimated by two methods. One was based on the determination of the extent of detachment of cultured cells from culture plates in the presence of the agents during a 3 h incubation. The other was based on the determination of the rate of release of LDH from the cultured cells in the presence of the agents. Caco-2 cells were seeded and cultured on 96- or 24-well tissue culture plates in the presence of DMEM containing 10% v/v fetal calf serum and 1% nonessential amino acid solution in an atmosphere of 95% air and 5% CO_2 at 37°C. When the cells were confluent, the cytotoxicity assays were performed.

In the cell detachment assay, the confluent cultured cells on 96-well plates were incubated in the presence of NO donors or other absorption enhancers for 3 h at 37°C, and then fixed with 4% glutaraldehyde and stained with 2% methylene blue (16). After washing 4 times with phosphate-buffered saline, the dye-bound cellular protein was extracted with 0.33 N HCl. Absorbance at 630 nm was measured using a 96-well microplate reader.

In the LDH release assay, the cultured Caco-2 cells on 24-well plates were added to the medium containing SNAP or sodium caprate (C10). At predetermined intervals of incubation at 37°C, the incubated medium was corrected and the LDH activity in the incubated medium was assayed using a kit (LDH-UV test-Wako). Maximal LDH release from the cells was determined by incubation of the cells in medium containing 0.1% Triton X-100.

Statistical Analysis

All results are expressed as mean values ± standard error (S.E.). Statistical analyses between two groups were performed using Student's *t*-test, and one-way analysis of variance (ANOVA) as appropriate. *P* values of 0.05 or less were considered to indicate statistically significant differences.

RESULTS

Effect of SNAP on Insulin Absorption

The coadministration of various doses of the NO donor, SNAP, markedly increased the rectal absorption of insulin (Fig. 1A). While insulin alone was poorly absorbed from the rectum, it was rapidly absorbed when coadministered with SNAP (4.0 mg, 18 μmol). Administration of a suppository containing SNAP (4.0 mg) without insulin had no effect on the plasma insulin level (data not shown). These findings suggest that SNAP did not induce release of insulin into the plasma from the body, and that the increase in the plasma insulin levels depended upon the enhancement of the rectal absorption of the bovine insulin.

Plasma Glucose Concentration

Figure 1B shows the plasma glucose concentration following rectal administration of the suppositories containing insulin and various doses of SNAP (Fig. 1B). Administration of a suppository containing SNAP without insulin had no effect on the plasma insulin level (data not shown). These findings suggest that the decrease in the plasma glucose concentrations was mediated by the insulin absorbed from the rectum. This also suggests that SNAP stimulated the absorption of the insulin from the rectal mucosa without loss of biological activity. The mean values of pharmacokinetic and pharmacodynamic parameters of the plasma insulin and glucose are summarized in Table 1. The AUC_{0-6} after the coadministration of SNAP (4 mg) was 4-fold higher than that in the controls. The increased AUC_{0-6} was dependent on SNAP dosage. When 1.0–4.0 mg of SNAP

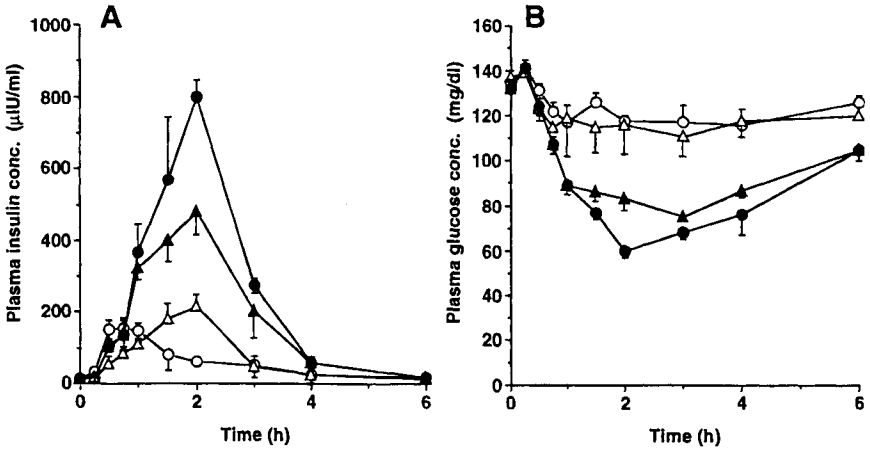

Fig. 1. Plasma insulin (A) and glucose (B) concentrations in rabbits following rectal administration of suppositories containing insulin (6.5 IU) and SNAP. Insulin without SNAP (○), insulin with 0.25 mg SNAP (△), insulin with 1.0 mg SNAP (▲), insulin with 4.0 mg SNAP (●). Each point represents the mean ± S.E. (vertical bar) for 3–5 rabbits.

was coadministered with insulin, significant differences were found in the AUC_{0-6}, Cmax and Tmax values for insulin and the Δ Glc value between the control group and the SNAP groups.

Absorption Enhancement Effects of Other NO Donors

To determine whether it was the SNAP or the NO derived from the SNAP which enhanced the insulin absorption in the rabbit rectums, we investigated whether other NO donors, NOR1 and NOR4 (17), stimulate insulin absorption from the rectum. NOR1 (4.2 mg, 18 μmol) enhanced insulin absorption (Fig. 2A). When the NOR4 (5.6 mg, 18 μmol), which produces NO more slowly than does NOR1 (18), was coadministered with insulin, no absorption enhancement effects were observed (data not shown). However, NOR4 (5.6 mg, 18 μmol) administered 2 h prior to the insulin enhanced the absorption of the insulin (Fig. 2A). The AUC_{0-6} values for the control, NOR1, and NOR4 cases were 337 ± 75, 1416 ± 364, and 902 ± 31 (h μIU/ml), respectively. Significant differences in AUC_{0-6} values were observed between the control case and both NO donors cases ($P<0.001$). Figure 2B shows the plasma glucose concentrations after coadministration of NOR1 or pretreated NOR4 with insulin.

Effect of NO Scavenger on Insulin Absorption

We also investigated the relationship between NO and enhancement of insulin absorption by an NO donor using an NO scavenger, carboxy-PTIO (Fig. 3 and Table 1). The enhancement of insulin absorption by SNAP (4.0 mg) was diminished by coadminstration of carboxy-PTIO (30 mg). The AUC_{0-6} values for insulin in the case of SNAP administration and in the case of SNAP administration with carboxy-PTIO were 1466 ± 174 and 506 ± 79 (h μIU/ml), respectively ($P<0.001$).

Effect of NO Donor on FD4 Absorption

Many investigators have reported that some protease inhibitors enhance mucosal absorption of polypeptides or proteins such as insulin (19,20). These inhibitors enhance the stability of proteins by reducing the activities of the mucosal proteases.

Table 1. Pharmacokinetic and Pharmacodynamic Parameters for Insulin and Glucose Following Rectal Administration of Insulin, SNAP, and Carboxy-PTIO

			Insulin			Glucose
	SNAP (mg)	Carboxy-PTIO (mg)	AUC_{0-6} (h μIU/ml)	Cmax (μIU/ml)	Tmax (h)	ΔGlc (h mg/dl)
Control	0	0	337 ± 75	168 ± 16	0.67 ± 0.14	78 ± 19
SNAP	0.25	0	432 ± 95	227 ± 15[a]	1.83 ± 0.29[a]	112 ± 30
	1.0	0	1059 ± 155[a]	457 ± 105[a]	2.00 ± 0.00[b]	233 ± 22[b]
	4.0	0	1466 ± 174[b]	797 ± 50[b]	2.00 ± 0.00[b]	279 ± 23[b]
Carboxy-PTIO	4.0	30	506 ± 79[c]	260 ± 72[c]	1.17 ± 0.29[c]	117 ± 70[d]

Note: Each value represents the mean ± S.E. for 3–5 experiments.
[a] $P < 0.01$ compared to control.
[b] $P < 0.001$ compared to control.
[c] $P < 0.001$ compared to SNAP 4.0 mg.
[d] $P < 0.005$ compared tp SNAP 4.0 mg.

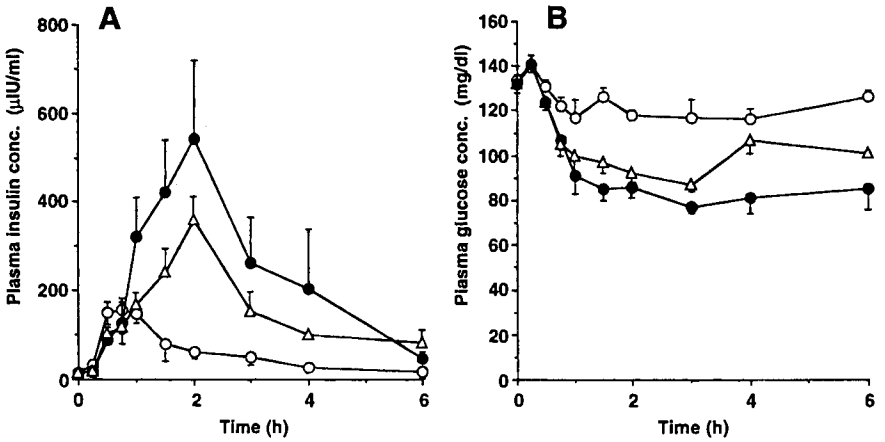

Fig. 2. Plasma insulin (A) and glucose (B) concentrations in rabbits following rectal administration of suppositories containing insulin (6.5 IU) and NOR1 or NOR4. Insulin without NO donor (○), insulin coadministered with 4.2 mg NOR1 (●), and insulin with 5.6 mg NOR4 (△) 2 h pretreated. Each point represents the mean ± S.E. (vertical bar) for 3–5 rabbits.

To determine whether the enhanced absorption by the NO donors was due to decreased protease activity, we determined whether an NO donor could enhance the absorption of FD4. This polysaccaride is poorly absorbed from the rectal mucosa and is not degraded by proteases. SNAP (4.0 mg) enhanced not only insulin absorption but also FD4 absorption (Fig. 4). The $AUC_{0\text{-}6}$ value for FD4 in the presence of SNAP was significantly higher than the $AUC_{0\text{-}6}$ value for FD4 without SNAP ($P<$ 0.001; SNAP; 27.87 ± 0.21 h ng/ml vs control; 0.90 ± 0.23 h ng/ml).

Cytotoxicity of NO Donors

The cytotoxicity of the NO donors and other absorption enhancers on the intestinal mucosa were evaluated by two methods. In the cell detachment assays, sodium caprate (C10) and heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CyD) which enhance rectal absorption of many drugs (21,22), were cytotoxic at 1 mg/ml or more (Fig. 5). The concentrations of

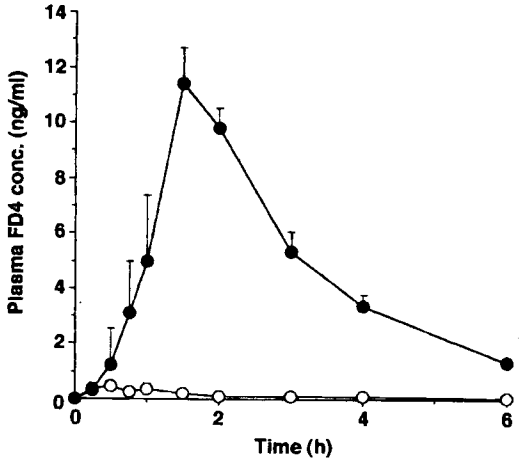

Fig. 4. Plasma FD4 concentration in rabbits following rectal administration of hollow-type suppositories containing FD4. FD4 (30 mg) without SNAP (○), FD4 with 4.0 mg of SNAP (●). Each point represents the mean ± S.E. (vertical bar) for 3–5 rabbits.

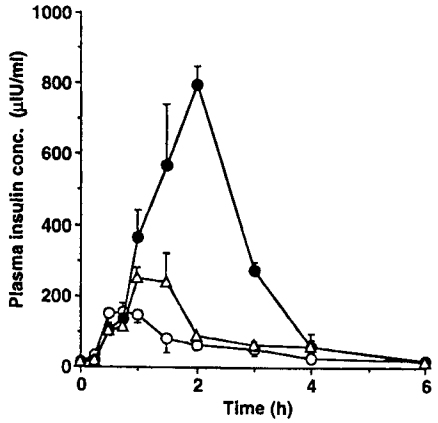

Fig. 3. Plasma insulin concentration in rabbits following rectal administration of suppositories containing insulin (6.5 IU), SNAP (4.0 mg) and carboxy-PTIO (30 mg). Insulin only (○), insulin with SNAP (●) and insulin, SNAP, and carboxy-PTIO (△). Each point represents the mean ± S.E. (vertical bar) for 3–5 rabbits.

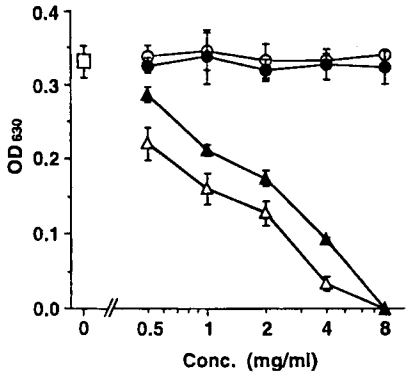

Fig. 5. Cytotoxicity of NO donors or absorption enhancers to Caco-2 cells. Caco-2 cells were cultured with or without the reagent (0.5, 1.0, 2.0, 4.0, and 8.0 mg/ml). After 3 h, the cells were fixed with glutaraldehyde and stained with methylene blue. The dye was extracted with 0.33 N HCl and the absorbance at 630 nm was measured. Control (□), SNAP (○), NOR1 (●), DM-β-CyD (△), and C10 (▲).

C10 and DM-β-CyD at which half of the cells detached from the culture plate during 3 h of incubation were 0.8 mg/ml and 2 mg/ml, respectively. However, neither SNAP nor NOR1 was cytotoxic at a concentration of less than 8.0 mg/ml. Figure 6 (A and B) shows the effect of C10 (A) or SNAP (B) on the rate of LDH release from Caco-2 cells. During incubation of the cells for 15 min in the presence of C10 (3.0 mg/ml), no LDH release was detected from the cells. However, during incubation of the cells for 2 h in the presence of only 0.1 mg/ml of C10, LDH release from the cells was detected. In contrast, 3.0 mg/ml of SNAP induced low-rate LDH release from the cells during 2 h of incubation. However, this LDH release rate was not significantly different from the rate of LDH release from the control cells. SNAP did not inhibit the activity of the LDH which was released (data not shown). Therefore, the low level of LDH release in the presence of SNAP depended on its low level of cytotoxicity to the Caco-2 cells.

DISCUSSION

The absorption of rectally administered hydrophilic drugs, such as peptides and proteins, is severely limited by the low permeability of the rectal mucosa to such compounds. Many efforts have been made to enhance the uptake of poorly absorbed drugs; coadminstration of an absorption enhancer is one of the most widely studied approaches. Numerous classes of compounds that enhance not only rectal but also intestinal uptake of hydrophilic drugs such as antibiotics, peptides, and proteins have been described (23). However, in most cases, the enhancement of drug absorption is accompanied by mucosal injury induced by the enhancer (4). Based on the results of the present studies, we propose that NO donors are potent absorption enhancers, and that their absorption enhancement mechanism may be a new one which is based on the intestinal epithelial actions of the chemical mediator, NO.

SNAP, NOR1, and NOR4 each enhanced insulin absorption from the rectum (Figs. 1 and 2), and coadminstration of an NO scavenger inhibited the absorption enhancement of the NO donor (Fig. 3). These findings strongly suggest that the NO derived from the NO donors enhanced the rectal permeability. The data in Fig. 4 suggest that the mechanism of absorption enhancement by NO did not depend on inhibition of protease activity in the rectal mucosa. Salzman *et al.* (13) reported the mechanism of the tight junction dilation by NO using cultured epithelial monolayers (Caco-2 cells). In this report, NO or NO donors increase the permeability of the Caco-2 monolayers, and the hyperpermeability was not due to loss of cell viability, as confirmed by intact ultrastructure, unaltered LDH release, and ability to recover baseline permeability. They also reported that NO donor led to decrease of cellular ATP levels, diminished fluoresceinphalloidin staining of junctional actin by cofocal microscopy observation, and widened tight junctions by electron microscopy observation. We have no data of actual mechanisms on the rectal absorption enhancement by the NO donors *in vivo*. It could be that NO acts by means of a chemical mediator to dilate tight junctions, conferring the enhancement of rectal absorption.

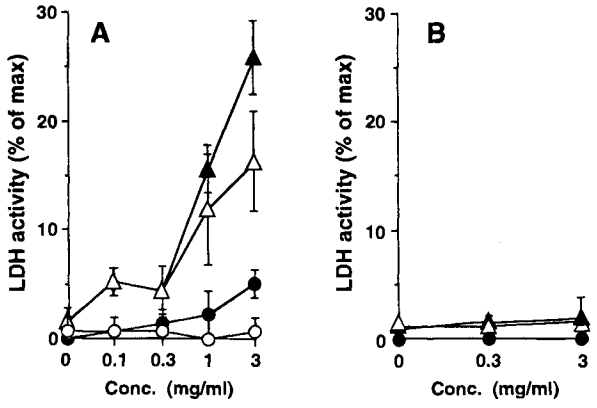

Fig. 6. Effect of C10 or SNAP on the LDH release from Caco-2 cells. Caco-2 cells were cultured in the presence of C10 (A: 0.1, 0.3, 1.0 and 3.0 mg/ml) or SNAP (B: 0.3 and 3.0 mg/ml). After various durations of culture (○, 15 min; ●, 30 min; △, 60 min; ▲; 120 min), the level of LDH activity in the culture supernatant was measured. Each value represents the % of maximal LDH release which was achieved by the conditioned medium of 0.1% Triton X-100 treated cells.

Many absorption enhancers stimulate drug absorption immediately (in many cases within 1 h). A new class of absorption enhancer, NO donors have a long time lag (Table 1; Tmax is 2 h) of absorption enhancement effect compared to the other types of enhancers. Many types of enhancers induce cell injury, providing the absorption enhancement effect (4). However, NO dilates tight junctions of epithelial monolayers and increases paracellular permeability without cellular injury (13). The difference of the time lag between NO donors and other enhancers may depend on the absorption enhancement mechanism.

The most serious problem related to use of absorption enhancers is mucosal damage. Some absorption enhancers exhibit cytotoxicity in the area of administration. Of these enhancers, C10 is a comparatively safe one, and is now clinically used in a commercial rectal suppository as an absorption enhancer of sodium ampicillin in Japan (Ampirect™, Kyoto Pharm. Ind., Co., Kyoto, Japan). The mechanisms of the absorption enhancement by C10 have been well studied (3,24–26). C10 induces paracellular expansion which is mediated by the increase in the intracellular calcium level, which leads to microfilament contraction in tight junctions. Tomita *et al.* discussed in their report the C10-induced increase in epithelial permeability by a physiological reaction of the cells (25). However, in our present studies, C10 exhibited cytotoxicity to the cultured intestinal epithelium in the assay of cell detachment from the culture plates and in the LDH release assays with long-term incubation (Figs. 5 and 6). The differences between our data and previously reported data may be due to differences in the experimental conditions. The change in intracellular Ca^{2+} level occurred rapidly, i.e., within 5 min. On the other hand, the cell detachment and LDH release induced by C10 occurred during long-term incubation (2 h). *In vivo*, the absorption enhancement effects of C10 or SNAP do not occur within a short time (5 min). Therefore, the main mechanism of the absorption enhancement by C10 may not depend on the physiological reaction of the cells. The enhancement of epithelial permeability by an NO donor is observed more than 4 h after the NO donor is added to the epithelial monolayers (18). The comparatively late occurrence (Tmax, 2.0 h) of the enhancement effect of SNAP *in vivo* is reasonable in relation to the *in vitro* results.

In the *in vivo* experiments, C10 or DM- β-CyD was used at 30 mg per rabbit for enhancement of rectal absorption (21,22).

The volume of rectal pituita in rabbits is less than 2 ml. If 30 mg of an absorption enhancer (C10 or DM- β-CyD) dissolved 2 ml of pituita, the concentration of the enhancer would be about 15 mg/ml immediately after administration, if the dissolution was rapid. This concentration of C10 or DM- β-CyD is much higher than the cytotoxic concentration for intestinal epithelium indicated by our data (Figs. 5 and 6). However, SNAP and NOR1 both enhanced the rectal absorption at only 4.0–4.2 mg per rabbit. If these NO donors dissolved into 2 ml of pituita, their concentration would be about 2 mg/ml. At this concentration neither of the NO donors exhibited cytotoxic effects. This suggests that these NO donors may enhance rectal epithelial permeability by means of an epithelial reaction without cell damage *in vivo*. In fact, our preliminary studies showed that NO donors had no serious rectal tissue injury in histochemical observation.

NO has many biological functions including acting as an important biological messenger related to many phenomena *in vivo*. NO stimulates insulin secretion from cultured pancreatic B cells (27,28). However, an NO donor inhibits insulin release from isolated pancreatic B cells (29). In our present studies, NO neither stimulated nor inhibited the secretion of insulin *in vivo* after administration of an NO donor to the rectum. The differences between our data and other reported data may be due to differences in the experimental conditions. For example, their data were obtained in studies in which cultured or isolated cells were used and the direct NO effect on pancreatic B cells was examined. However, we used whole animals and administered NO donors to rectal mucosa *in vivo*.

An ideal absorption enhancer would have no biological side effects, and only enhance the absorption of target drugs. We have no data on the side effects of NO donors used as absorption enhancers in the rectum. Because of the short half-life of NO gas in solution (3-5 s), NO donors may act mainly at the site of their administration in the mucosa, leading to few side effects occurring elsewhere in the body.

In conclusion, we propose that NO donors are potent absorption enhancers, and exert this effect via a previously unidentified absorption enhancement mechanism based on the dilation of tight junctions by means of an epithelial reaction.

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