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Nicotinamide inhibits sodium-dependent phosphate cotransport activity in rat small intestine

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Abstract

Background. We recently reported that the administration of niceritorol (a nicotinic acid derivative which improves lipid metabolism and peripheral circulation, and is used for the treatment of hyperlipidaemia and impaired peripheral circulation) to patients with hyperphosphataemia undergoing dialysis decreased the serum phosphate (Pi) concentration. We found that this was due to an acceleration of faecal Pi excretion by niceritrol.

Methods. Intestinal brush border membrane vesicles (BBMVs) were prepared from rat jejunum, and the Na^+-dependent and Na^+-independent Pi transport activities in these vesicles were measured. In addition, the functional Pi transporter from rat small intestine was injected in *Xenopus* oocytes, and the effect of nicotinamide on the levels of its expression were measured by northern blotting.

Results. The Na^+-dependent component was significantly decreased in the BBMVs isolated from rats treated with nicotinamide, while the Na^+-independent component was not changed. Kinetic studies demonstrated that the decreased activity was due to reduction of the V_{max} value and not an elevation of the K_m values. When poly(A)$^+$RNA from rats treated with nicotinamide was microinjected into *Xenopus* oocytes, the Pi transport activity was significantly decreased compared with that in the control animals. In addition, there were no significant changes in Na/Pi cotransporters and activators, but the vitamin D receptor mRNA level was reduced to 80% of the control level.

Conclusions. These observations suggest that nicotinamide may regulate the expression of a major functional Na/Pi cotransporter in the rat small intestine.

Key words: nicotinamide; phosphate; small intestine

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Introduction

In chronic renal failure (CRF), phosphate (Pi) retention may lead to secondary hyperparathyroidism and a progression to end-stage renal disease [1]. Dietary Pi restriction or Pi binders can slow the progression of experimental CRF [2]. Conversely, a diet high in Pi can accelerate the progression toward end-stage renal disease [1,2]. The current therapy for hyperphosphataemia in patients with CRF consists of the administration of Pi binders. Pi binders reduce intestinal Pi absorption, but have several side effects. New and safe drugs which can inhibit intestinal Pi transport are needed, and the molecular structure and regulation of intestinal Pi transport should thus be elucidated.

Niceritorol is a nicotinic acid derivative which improves lipid metabolism and peripheral circulation, and is used for the treatment of hyperlipidaemia and impaired peripheral circulation. We reported that the administration of niceritorol to patients undergoing dialysis for the treatment of high-density lipoprotein (HDL) hypocholesteraemia caused a reduction of the serum Pi concentration [3]. Our results indicated that an acceleration of faecal Pi excretion might be involved the mechanism underlying the reduction of the serum Pi concentration which is observed in patients undergoing dialysis. On the basis of these observations, we concluded that nicotinamide significantly decreases intestinal Pi transport activity.

The intestinal Pi transport process occurs by both a sodium-independent, nonsaturable process and via an active, sodium-dependent component of phosphate absorption, mainly in the duodenum and jejunum [4]. However, the regulation of intestinal Pi transport is not well understood, in part because of the lack of knowledge about the structure of intestinal Na/Pi cotransporters [5]. Three types of Na/Pi cotransporter have been isolated from several species [6–8]. The type I and type II Na/Pi cotransporters are expressed mainly in renal epithelial cells [6]. Type III transporters are widely expressed in mouse, rat and human tissues

[7]. Type III transporters were isolated as receptors for gibbon ape leukemia virus (GLVR1 or PiT-1) in mice and humans and amphotropic murine retrovirus (Ram-1 or PiT-2) in rats [7], and were shown to have normal cellular functions as Na^+-dependent Pi cotransporters in several tissues. The amino acid sequences of PiT-1 and PiT-2 transporters are approximately 60% identical [7], and exhibit no significant overall sequence homology with the type I or type II transporters.

In the rat small intestine, a putative activator protein (PiUS) for the Na^+-dependent Pi transporter was reported [9]. PiUS was cloned from the rabbit small intestine by expression cloning. PiUS markedly stimulated Na/Pi cotransport activity in *Xenopus* oocytes [9]. The putative amino acid sequence of PiUS cDNA revealed a highly hydrophilic protein and no membrane spanning domain, suggesting that PiUS may be an activator for the Na^+-dependent Pi transporter [9]. In addition, in the rat and human brains, a family of brain-specific Na/Pi cotransporters (BNPI) was cloned and were also found to be expressed in the small intestine [8]. However, their functional roles and regulation are unknown. In the present study, we investigated the effect of nicotinamide on intestinal Na/Pi cotransporters.

Subjects and Methods

Animal preparations

Wistar strain rats (200 g) were (SLC, Shizuoka, Japan) kept in plastic cages and fed a standard laboratory chow (0.6% Pi) (Oriental Yeast, Tokyo, Japan). All rats were allowed unlimited access to food and distilled water. On the sixth day, the animals were intraperitoneally (i.p.) injected with either nicotinamide dissolved in isotonic saline (pH adjusted to 7.0 with NaOH) (4 mmol/kg body weight) or vehicle (0.9% NaCl). After 12 h the rats were administered nicotinamide or saline again. On the seventh day (24 h after first administration of nicotinamide or saline), the rats were sacrificed and the jejunum was rapidly removed. One-fourth of each jejunum was used for RNA isolation, and the other three-fourths were used for the isolation of brush border membrane vesicles (BBMVs).

Preparation of BBMVs and transport measurements

BBMVs were prepared from the rat small intestine (jejunum) by the Ca^{2+} precipitation method, as described previously [10]. The purity of the membranes was assessed by measuring the levels of leucine aminopeptidase, (Na^+K^+) ATPase and cytochrome-c oxidase [10]. The uptake of radiolabelled Pi was measured by the rapid filtration technique [10]. After 10 µl of the vesicle suspension was added to 90 µl of the incubation solution (100 mM NaCl, 100 mM mannitol, 20 mM HEPES–Tris, 0.1 mM KH_2PO_4), the preparation was incubated at 20°C. The measurements of Na^+-dependent and Na^+-independent Pi uptake were performed as described previously [10]. Transport was terminated by rapid dilution with 3 ml of an ice-cold solution (100 mM mannitol, 20 mM HEPES–Tris, 0.1 mM KH_2PO_4, 20 mM $MgSO_4$ and 100 mM choline chloride). The reaction mixture was then immediately transferred to a premoistured filter (0.45 µm) maintained under a vacuum.

The measurement of Pi transport in microinjected Xenopus *oocytes*

Xenopus laevis females were obtained from Hamamatsu Jikken (Shizuoka, Japan) [11]. Small clumps of oocytes were treated twice for 45 min each time with collagenase (2 mg/ml) in Ca^{2+}-free solution (OR II solution: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES–Tris, pH 7.5) in order to remove the follicular layer. After extensive washing, first with OR II solution and then with modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES–Tris, pH 7.5), the oocytes were maintained in modified Barth's solution overnight at 18°C. Healthy stage-V oocytes were then injected with cRNA (2.5–5 ng of cRNA in 50 nl) with the use of a digital misrodispenser followed by incubation at 18°C for 3 days, and the Pi transport activity was measured. For the measurement of Pi uptake, oocytes were first washed for 30 s with solution A (100 mM sodium chloride or 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES–Tris, pH 7.5), and groups of six or seven oocytes per condition were then incubated at 18°C for 60 min in the same medium containing 0.1 mM $KH_2{}^{32}PO_4$ (9000 Ci/ml)(NEN/Dupont, Boston, MA) [11]. The measurements of Na^+-dependent and Na^+-independent Pi uptake were performed as described previously [11]. After being washed three times with ice-cold solution A containing 5 mM KH_2PO_4, each oocyte was transferred to a scintillation vial and dissolved in 0.2 ml of 10% (w/v) sodium dodecyl sulfate (SDS); the oocyte-associated radioactivity was the measured by scintillation spectroscopy after the addition of 5 ml of scintillation fluid [11].

Northern blot analysis

Total RNA from the rat small intestine (jejunum) was isolated by extraction with acid guanidine thiocyanate/phenol/chloroform using the method of Chomczynski *et al.* [12]. Poly(A)$^+$ RNA isolated using an oligo-dT column was denatured by being heated at 70°C in 10 mM MOPS, pH 7.0, containing 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, and 50% (v/v) formamide for 5 min and subjected to electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde. Resolved RNA was transferred to a Hybond-N membrane (Amersham, Buckinghamshire, UK) and covalently cross-linked by exposure to UV light. Hybridization was performed in a solution containing 50% formamide, 5 × SSPE, 2 × Denhardt's solution, and 1% SDS. The membranes were exposed in a bio-imaging analysis system (BAS1500, Fuji Photo Film Co., Tokyo). Total RNA from various rat tissues was denatured, fractionated by electrophoresis on a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane, and subjected to hybridization with randomly primed, ^{32}P-labelled rat PiT-1, rat PiT-2 [7], rat BNPI [8], rat PiUS [9], rat VDR [13], rat calbindin D-9K [13], rat Na/K ATPase [14], rat sodium-dependent glucose transporter SGLT1 [10], or rat peptide transporter PepT1 [15].

Hybridization to the labelled probes was performed overnight in a solution of 50% deionized formamide, 10 × Denhardt's solution, 40 mM Tris–HCl (pH 7.5), 10 mg/ml salmon sperm DNA and 1% SDS at 42°C. The

membranes were washed twice for 10 min each time with 0.1 × SSC/0.1% SDS at 60°C (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). The filters were exposed for 2, 12 and 24 h to a Bio-Imaging Plate and quantified by the BAS1500 system mentioned above.

Serum measurements

Plasma Ca^{2+} were measured using OCPC method (Wako, Osaka, Japan). Plasma Pi was measured with a p-methelaminophenol-based reagent kit (Wako, Osaka, Japan). Plasma 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) levels were measured by a radioreceptor assay (Incstar, Minneapolis, MN). Plasma intact parathyroid hormone (PTH) levels were measured with a rat immunoradiometric assay (Nichols, San Clemente, CA).

Statistical analysis

Values are expressed as mean ± standard error (SE). The differences between the means of two groups and three or more groups were estimated by Student's *t*-test and one-way analysis of variance (ANOVA), respectively. A P value of <0.05 was considered significant.

Results

Effects of nicotinamide on intestinal Na/Pi cotransport activity

The Na^+-dependent Pi uptake was determined in the isolated BBMVs from the jejunum of normal rats. The Pi uptake was linear up to 30 s and slowly increased to 5 min. In comparison to the isolated BBMVs from rat kidney cortex, the total Pi uptake in the intestinal Pi transport was lower than that in the kidney. Next, we examined whether nicotinamide affects the intestinal Pi transport activity (Figure 1A). We measured the Na/Pi cotransport activity in the BBMVs isolated from the rats treated with nicotinamide (4 mmol/kg body weight). The initial Na^+-dependent Pi cotransport activity (at 15 s) in the rats treated with nicotinamide was decreased by about 2-fold compared with that in the rats injected with saline (controls). The Na^+-dependent Pi transport component was estimated to be about 50% of the total Pi uptake at 15 s. The Na^+-dependent component was significantly decreased in the BBMVs isolated from rats treated with nicotinamide, while the Na^+-independent component was not changed significantly (Figure 1B). The reduction of the Na/Pi cotransport activity was observed 2 days after the administration of the drug, and the maximal effect was shown in 1 day after administration. However, the plasma Pi concentration was not reduced in the rats at 12 or 24 h after the administration of nicotinamide (Table 1). In addition, we found that the level of serum 1,25-$(OH)_2D_3$ was slightly decreased in the nicotinamide-treated rats (Table 1).

In the rats treated with nicotinamide for 24 h, kinetic studies demonstrated that the decreased activity was due to a reduction of the V_{max} value (control; 72 ± 10 pmol/mg protein/10 s *versus* nicotinamide; 22 ± 4 pmol/mg protein/10 s, and not an elevation of the K_m values (control; 0.12 ± 0.02 mM *versus* nicotinamide; 0.12 ± 0.03 mM) (Figure 2).

In addition, the intestinal uptakes of [^{14}C]-L-leucine, [^{14}C]-D-glucose, and [^{3}H]alanine were not significantly changed in the rats treated with nicotinamide (Figure 3).

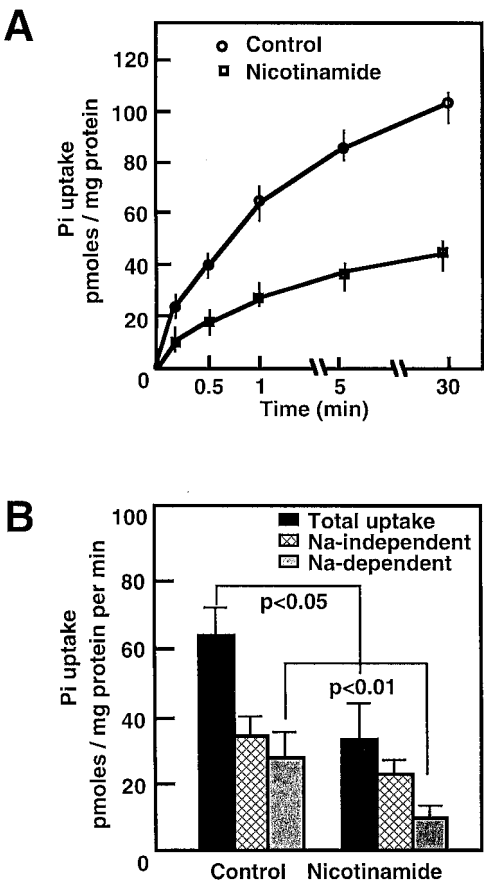
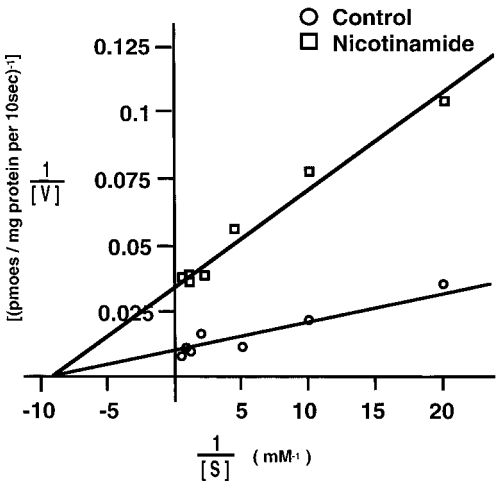


Fig. 1. Effects of nicotinamide on intestinal Pi transport in the BBMVs. (**A**) Time course of Pi uptake in the BBMVs isolated from normal or nicitinamide-treated rats. Intestinal BBMVs were isolated from rats 24 h after the administration of nicotinamide. Pi transport activity was measured at the indicated times. (**B**) Comparison of the Na^+-dependent and Na^+-independent components of the Pi uptake. Na^+-dependent and Na^+-independent Pi transport activities were measured at 1 min as described in Materials and Methods. Data are means ± SE ($n=6$, each group).

Table 1. Effects of nicotinamide on plasma Ca^{2+}, Pi, PTH, and 1,25$(OH)_2D_3$ levels

	Control	Nicotinamide (24 h)	
Ca^{2+} (mg/dl)	8.47 ± 0.32	7.83 ± 0.28	NS
Pi (mg/dl)	7.25 ± 0.29	6.82 ± 0.30	NS
PTH (pg/ml)	30.4 ± 4.51	36 ± 5.33	NS
1,25$(OH)_2D_3$ (pg/ml)	65.0 ± 4.5	48.2 ± 3.6	$P<0.05$

Results are means ± SEM ($n=5$). $P<0.05$ *versus* corresponding control.



	Vmax (nmoles / mg protein / 10sec)	Km (mM)
Control	72 ± 10[a]	0.12 ± 0.02
Nicotinamide	22 ± 4[a]	0.12 ± 0.03

[a] $p < 0.01$ $n = 3$.

Fig. 2. Kinetic analysis of Pi transport activity (**A**) Lineweaver-Burk plot of [^{32}P]KH_2PO_4 transport by jejunum BBMVs isolated from control and nicotinamide-treated rats. Pi transport activity was measured at concentration 0.01–10 mM of KH_2PO_4. (**B**) V_{max} and K_m for Pi uptake. Values are means ± SE ($n = 3$, each group).

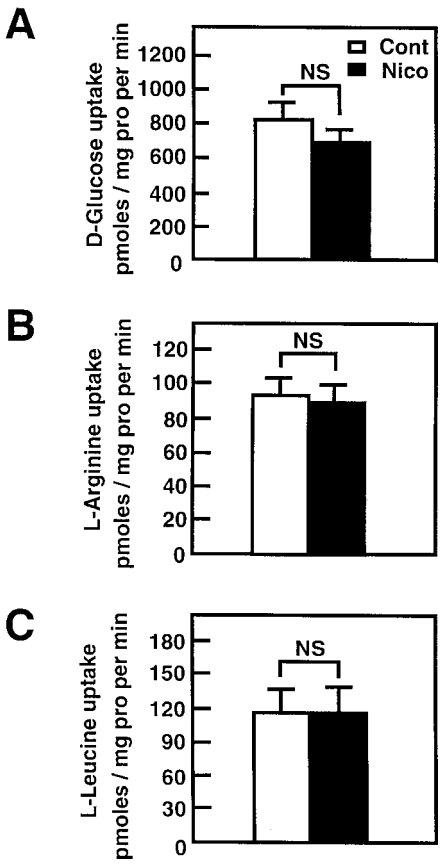


Fig. 3. Glucose and amino acid transport activities in the BBMVs isolated from control and nicotinamide-treated rats. Twenty-four hours after the first administration of nicotinamide to rats, jejunal BBMVs were isolated as described previously [11]. (**A**) In the presence of Na^+, D-[^{14}C]glucose (0.1 mM), (**B**) [^{3}H]-L-arginine (50 μM), and (**C**) L-[^{14}C]leucine transport activities were measured as described previously [11]. Values are means ± SE ($n = 5$, each group).

Functional analysis of Na/Pi cotransport activity in Xenopus *oocytes*

To further analyse the effect of nicotinamide on Na/Pi cotransport activity, we isolated poly $(A)^+$RNA from normal rat jejunum and microinjected it into *Xenopus* oocytes. As shown in Figure 4A, the Na^+-dependent Pi cotransport activity was increased by about 2-fold compared with that in water-injected controls. In contrast, when poly$(A)^+$RNA from rats treated with nicotinamide was microinjected into *Xenopus* oocytes, the Pi transport activity was significantly decreased compared with the activity in the control animals, and the levels of Na/Pi cotransport were similar to those in the water-injected controls (A). In addition, the uptake of [^{14}C]-L-leucine were not significantly changed in *Xenopus* oocytes microinjected with poly $(A)^+$RNA isolated from the rats treated with nicotinamide (Figure 4B). These observations indicate that the amounts of functional intestinal Na/Pi cotransporter mRNA is reduced by the administration of nicotinamide.

Expression of intestinal Pi cotransporters

To investigate the expression of Na/Pi cotransporter genes in the rat small intestine, we subjected rat intestinal total RNA to a northern blot analysis with cDNA probes for the following Na/Pi cotransporters and activator: rat RNaPi-1, rat NaPi-2, rat PiT-1, rat PiT-2, rat BNPI, and rabbit PiUS. We found that PiT-1, PiT-2, and BNPI were expressed in the rat small intestine. In contrast, rabbit PiUS cDNA hybridized to several transcripts (Figure 5).

Effect of nicotinamide on the expression of Na/Pi cotransporters

To further investigate the expression of Na/Pi cotransporters 24 h after the administration of nicotinamide to the rats, a northern blot analysis was carried out. As shown in Figure 6A and B, the PiT-1, PiT-2, BNPI, PiUS, Na/K ATPase PepT1 and SGLT1 mRNA levels were not changed by nicotinamide. In contrast, we found that the vitamin D receptor mRNA level was reduced to 80% of the control level. Calbindin-D9k mRNA also decreased in the rats (Figure 7).

Discussion

In the present study, we investigated the effect of nicotinamide on rat intestinal Pi uptake. The intestinal Na/Pi cotransporter has properties that are similar to

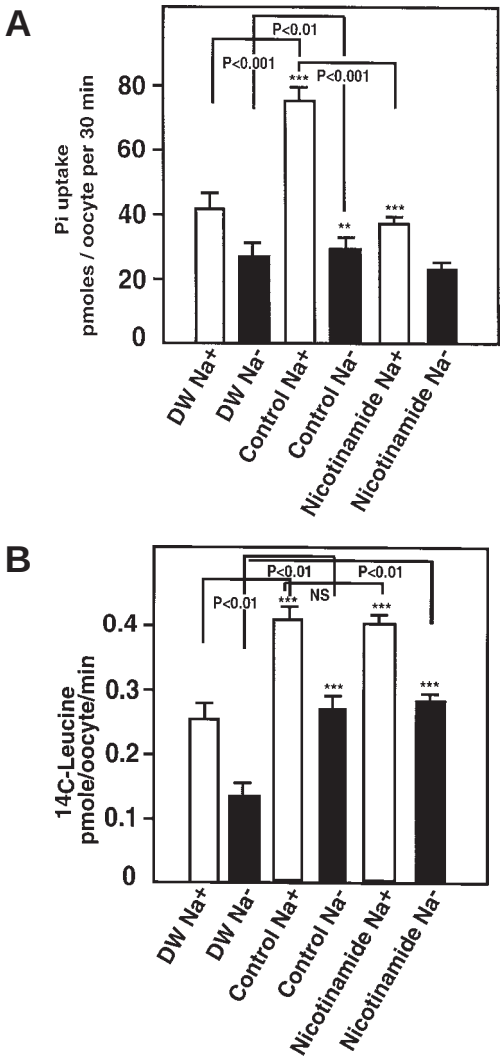


Fig. 4. Functional expression of jejunal poly(A) RNA isolated from control and nicotinamide-treated rats. The microinjection of poly(A)RNA (50 ng) into *Xenopus* oocytes was performed as described in Materials and Methods. (**A**) The Pi transport activity was measured at 18°C in the presence and absence of sodium chloride. Control oocytes were microinjected with distilled water (50 nl). Values are means ± SE ($n = 12$, each group). (**B**) The uptake of [^{14}C]leucine (50 μM) was measured in *Xenopus* oocytes injected water and poly(A)RNA 4 days after injection. Values are means ± SE ($n = 7$, each group).

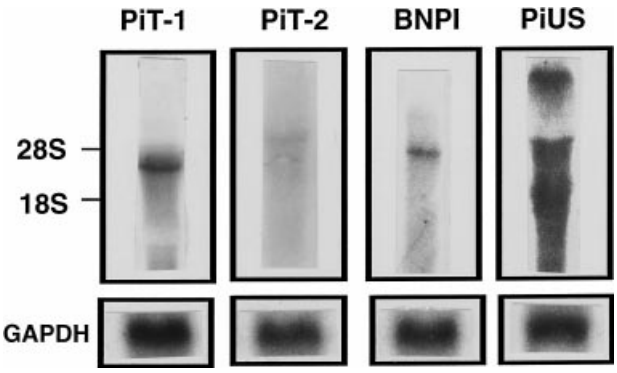


Fig. 5. Expression of Na/Pi cotransporters in rat small intestine. Total RNA (20 μg) isolated from rat jejunum was loaded on 1.2% argarose gels. The hybridization of each cDNA probes was performed as described previously. Rat type III Na/Pi cotransporter PiT-1, rat type III Na/Pi cotransporter PiT-2 (7), rat brain Na/Pi cotransporter BNPI (8), and rabbit Pi transporter activator protein PiUS (9) were used.

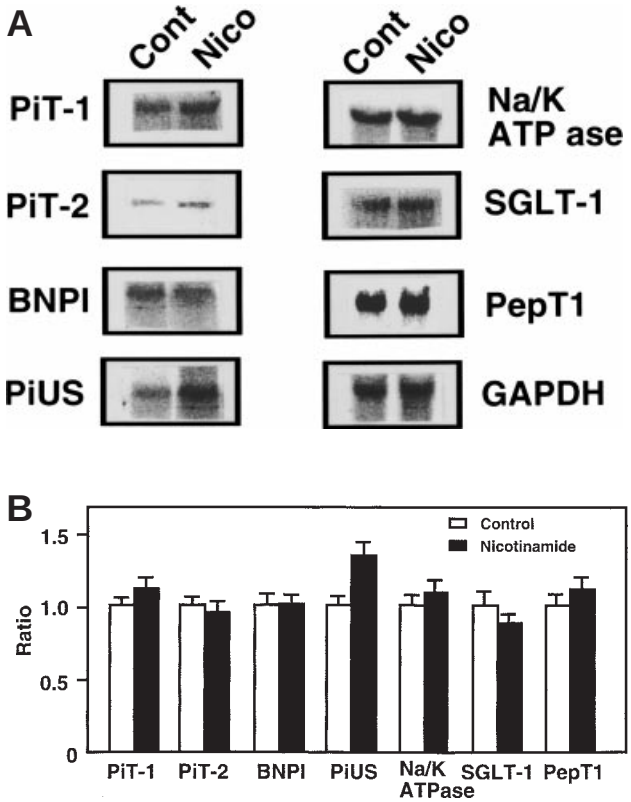


Fig. 6. Effects of nicotinamide on the expression of Na/Pi cotransporters in the jejunum. (**A**) Northern blot analysis. (**B**) Relative intensity of Na/Pi cotransporter mRNAs. The amounts of each transcript are represented as the relative intensity corrected by GAPDH. For the cDNA probes, rat Na/K ATPase (14), rat sodium-dependent glucose transporter SGLT1 (10), or rat peptide transporter PepT1 (15) were used. Values are means ± SE ($n = 5$, each group).

those of the renal Na/Pi cotransporter. However, several differences exist between these two transport systems. In contrast to the kidney, where the Na^+-independent component of Pi transport is negligible, this component comprises a much higher portion of the intestinal Pi transport. The Na^+-independent, diffusional component of intestinal Pi transport represents approximately 40–50% of the total uptake. This component is significantly higher in the rat ileum than in the jejunum (data not shown). In addition, the capacity of the intestinal Na/Pi cotransport, as measured in rat jejunal BBMVs, was found to be significantly lower than that measured in renal BBMVs. These observations are consistent with those of other investigators [5,16]. In the kidney, at least three types of Na/Pi cotransporters have been isolated, and the properties of the transporters were well characterized. In

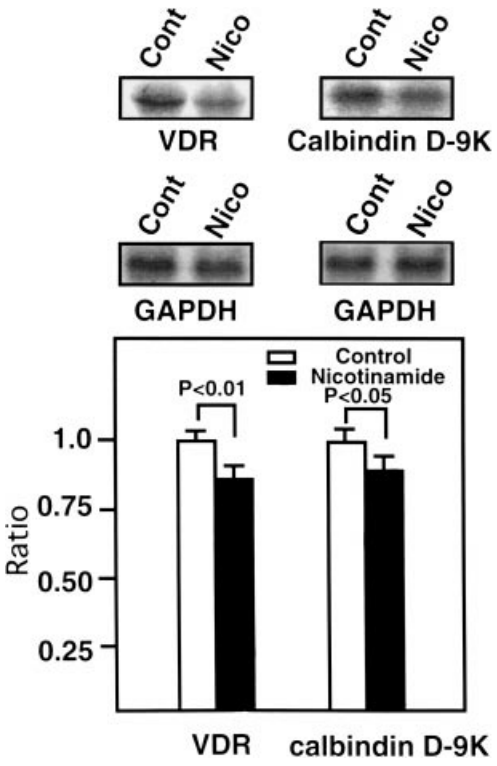


Fig. 7. Effects of nicotinamide on the expression of vitamin D receptor (VDR) and calbindin-D9k mRNA in the jejunum. Total RNA (20 μg) was isolated from the jejunum of rats treated with nicotinamide as described in Figure 5. (**A**) Northern blot analysis of VDR and calbindin-D9k mRNA transcript. (**B**) Relative intensity of each transcript corrected by GAPDH mRNA. Values are means ± SE ($n = 5$, each group).

contrast, a major functional Na/Pi cotransporter in the small intestine has not been identified.

To characterize the expression of the Na^+-dependent Pi transporter, we used total mRNA from jejunal mucosa and performed a kinetic analysis. In water- and mRNA-injected *Xenopus* oocytes, similar K_m values for Pi interaction (approximately 0.1 mM) were observed which are close to the K_m values reported for Na/Pi cotransport in isolated rat jejunum BBMVs (0.1–0.2 mM). In this context, we suspect that the rat PiUS can stimulate a major functional Na/Pi cotransporter in the small intestine, in addition to the endogenous Na/Pi cotransporter in *Xenopus* oocytes.

Niceritorol is a nicotinic acid derivative which improves lipid metabolism and peripheral circulation, and is used for the treatment of hyperlipidaemia and impaired peripheral circulation. A reduction of the serum Pi concentration by niceritorol has recently been noted in patients undergoing dialysis. In a previous study, we examined the effects of an oral administration of niceritorol to normal rats on faecal and urinary Pi excretion, the faecal Pi excretion significantly increased, and the body accumulation of Pi was significantly suppressed by niceritorol [3]. There have been no reports showing that nicotinic acid or its derivative increase faecal Pi excretion in rats. The mechanism of the increase in the faecal Pi excretion by niceritorol may be due to an inhibition of the Pi absorption in the small intestine.

Nicotinamide, a derivative of niceritorol, is a potent specific inhibitor of the renal proximal tubular transport of Pi [17]. Over a period of several hours, a single dose of nicotinamide produces an increase in NAD content in proximal tubules, a specific inhibition of the Na^+-dependent phosphate transport across the BBM of the proximal tubules and a marked increase in the urinary excretion of Pi [17]. In view of the many similarities and also some differences of Na/Pi cotransporters in the BBM of the kidney and intestine [18], we observed a similar effect of nicotinamide on Pi uptake by BBMV isolated from the rat small intestine. The molecular mechanisms underlying the inhibition of renal Pi transport have been well studied in the rat and mouse kidney. Dousa and Kempson suggested that nicotinamide stimulated the production of cAMP levels in the proximal tubular cells, in a mechanism very similar to that of the parathyroid hormone (PTH).

In the present study, we found that nicotinamide also decreased the VDR mRNA levels in the small intestine. The mechanism of the reduction of VDR mRNA levels are controlled by several factors, including the serum 1,25-$(OH)_2D_3$ and PTH levels and a low Pi diet. We found that the levels of serum 1,25-$(OH)_2D_3$ were slightly decreased in the nicotinamide-treated rats. The intestinal absorption of Pi is enhanced by vitamin D metabolites and specifically by 1,25-$(OH)_2D_3$, which also increases calcium absorption [19]. It is possible that the decrease of plasma $1,25(OH)_2D_3$ level may affect intestinal Na/Pi cotransport activity. However, we did not find any significant change in plasma calcium and PTH levels in the rats treated with nicotinamide.

The activities of Pi transport in the rat small intestine have been studied in vitamin D-deficient rats. The dependency of $1,25(OH)_2D_3$-mediated intestinal Pi transport on protein synthesis was shown by the inhibitory effect of cyclohexmide [20]; in a sense, this confirmed the finding of Ferraro *et al.* that the maintenance of the Pi absorption system of the intact rat is dependent on continuous protein synthesis [20]. However, in the present study, we did not find any fluctuation in the intestinal Na/Pi cotransporter mRNA levels. Further studies are needed to clarify the roles of VDR in the regulation of intestinal Na/Pi cotransport by $1,25(OH)_2D_3$.

Finally, we concluded that nicotinamide caused the decrease in the amount of the major functional transporter in the rat small intestine. The levels of several Na/Pi cotransporters and activator protein (PiUS) mRNAs were not changed in the rats treated with nicotinamide. In addition, the vitamin D receptor and calbindin-D9k mRNA levels were significantly decreased in those animals. These observations suggest that nicotinamide inhibits Na/Pi cotransport activity in the rat intestine, in addition to that in the renal proximal tubules.

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