Nicotinamide prevents the development of hyperphosphataemia by suppressing intestinal sodium-dependent phosphate transporter in rats with adenine-induced renal failure

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Abstract

Background. Nicotinamide has been shown to inhibit intestinal sodium-dependent phosphate transport activity in normal rats. It was reported recently that type IIb sodium-dependent phosphate co-transporter (NaPi-2b) is a carrier of intestinal phosphate absorption, and that its expression level is regulated by serum 1,25-dihydroxyvitamin D [1,25(OH)2D] and Pi levels in normal rats. However, in chronic renal failure (CRF), serum 1,25(OH)2D and Pi levels are often abnormal. In a rat model of CRF, we investigated whether short-term nicotinamide administration was effective in reducing intestinal phosphate absorption and, if so, whether the effect was mediated by intestinal NaPi-2b.

Methods. Adenine-induced CRF rats were given a single daily intraperitoneal administration of nicotinamide or vehicle solution for 6 days, and time course changes in serum Pi, Ca, blood urea nitrogen (BUN) and creatinine levels were monitored. Intestinal phosphate absorption was examined by oral administration of radiolabelled phosphate on the final day. In addition, NaPi-2b protein content in jejunum brush border membranes was determined.

Results. Nicotinamide prevented the progressive increase in serum Pi associated with renal failure and significantly inhibited intestinal Pi absorption as assessed by the influx of orally administered radio-labelled phosphate into the circulation. This effect was accompanied by a decrease in NaPi-2b expression in jejunum brush border membranes. In addition, nicotinamide treatment was also associated with less marked elevations in BUN and serum creatinine and a higher creatinine clearance.

Conclusions. Nicotinamide inhibited intestinal Pi absorption in a rat model of CRF, at least in part by inhibiting the expression of NaPi-2b, and appeared to protect against the deterioration of renal function.

Keywords: chronic renal failure; hyperphosphataemia; intestinal phosphate absorption; NaPi; nicotinamide

Introduction

Hyperphosphataemia during chronic renal failure (CRF) is not only a potential pathogenic cause of secondary hyperparathyroidism, but may also accelerate the progression of renal insufficiency towards end-stage renal disease [1,2]. This latter role has been supported by studies showing that dietary Pi restriction prevents the progression of CRF [3–6], whereas Pi supplementation accelerates renal failure [3,6]. Current therapy for hyperphosphataemia accompanying CRF includes the administration of Pi binders such as calcium carbonate, calcium acetate and aluminium-based compounds; however, these treatments have been limited by side effects (hypercalcaemia) and outright toxicity (aluminium). As a result of numerous attempts to develop alternative phosphate binders, a novel phosphate-binding polymer that contains neither aluminium nor calcium (Renagel®; sevelamer hydrochloride) has come into clinical use recently. However, use of this polymer has occasionally been associated with poor patient compliance owing to the high number of tablets required and need for administration before or with a meal, leading to the search for new drugs having fewer side effects and better compliance.

Niceritrol is a nicotinic acid prodrug that is clinically used to improve lipid metabolism and peripheral circulation in patients with hyperlipidaemia. Shimoda et al. [7] have reported that niceritrol decreased serum Pi concentration in patients undergoing dialysis, suggesting that it may also affect the
regulation of phosphate metabolism. Takahashi et al. [8] observed similar effects while using nicotinamide, a nicotinic acid derivative. Because Pi homeostasis is primarily maintained by a balance between intestinal Pi absorption and renal Pi excretion, it is possible that the hypophosphatemic effect of nectaritol is due to its inhibition of intestinal Pi absorption [9]. Katak et al. [10] reported that nicotinamide inhibits sodium-dependent Pi co-transporter activity in normal rat small intestine; however, they did not investigate its effects on urinary Pi excretion.

The molecular mechanisms underlying intestinal Pi absorption are not fully understood. However, a type IIb sodium-dependent Pi co-transporter (NaPi-2b) has been identified recently which actively transports phosphate on the intestinal brush border membrane [11]. There is evidence that intestinal NaPi-2b plays an important role in intestinal Pi absorption [11,12], and that it is regulated by serum Pi [13,14] or 1,25-dihydroxyvitamin D [1,25(OH)2D] levels [15]. However, the role that NaPi-2b plays in the development of abnormal phosphate and vitamin D metabolism associated with CRF, which is characterized by elevated serum phosphate and decreased serum 1,25(OH)2D levels, remains unclear.

In the present study, we examined the short-term effects of nicotinamide on serum Pi concentration, intestinal Pi absorption and urinary Pi excretion in a rat model of CRF. We additionally examined changes in NaPi-2b expression to better understand the inhibitory effect of nicotinamide on intestinal Pi absorption. Finally, we examined a possible protective effect of nicotinamide on the progression of renal function impairment in CRF.

Materials and methods

Experimental protocol

Male Wistar rats aged 6 weeks were purchased from Charles River Japan. They were fed a standard powder diet containing 0.85% Pi, 1.12% Ca, 25.3% crude protein and 2.5IU/g vitamin D3 (CE-2, CLEA Japan) and given free access to food and water. After an acclimatization period of 7 days, they were divided into two groups. The first group continued to receive the same diet without treatment until the end of the study (control group, \( n = 10 \)). The second group (\( n = 32 \)) was switched to a powder diet containing 0.75% adenine. At 14 days after the first feeding of adenine, these rats were again divided into two groups (\( n = 16 \) each) that were matched for body weight, serum Pi, Ca, blood urea nitrogen (BUN) and creatinine. On the following day (day 0), daily intraperitoneal injections were started with nicotinamide dissolved in saline (4 mmol/day/kg body weight, pH adjusted to 7.0 with NaOH) or vehicle (0.9% NaCl saline) for a total of 6 days. Ten rats each from the nicotinamide and vehicle groups were housed separately in metabolic cages for collection of 24 h urine on days 5-6. They were then sacrificed on day 6 for preparation of jejunal and renal brush border membranes (BBMs) on day 6. Body weight and food intake volume were measured daily, and blood samples were collected on days 0, 4 and 6 from the tail artery. The control group rats were also sacrificed on day 6. Blood samples were collected from the tail artery and jejununal and renal BBMs were prepared.

Serum and urinary variables

Serum Pi, Ca, glucose, creatine, BUN and urinary Pi were measured using an automatic serum analyser (Hitachi, Japan). Serum 1,25(OH)2D was measured with a radio receptor assay kit (SRL, Yamasa, Tokyo, Japan).

Measurement of blood radioactive phosphate for the estimation of intestinal Pi absorption

On day 6, 1 ml of liquid food (CLEA Japan) containing 4.28 mg of K2HPO4 and 27.2 \( \mu \)g of KH2PO4 radiolabelled phosphorus (NEN Lifescience Product, UK) was administered to the rats by oral gavage. At 45 min after administration, peripheral blood was collected from the tail vein and the radioactivity in 100 \( \mu \)l samples was determined using a liquid scintillation counter (Packard Japan).

Measurement of radioactive phosphate clearance

On day 6, 2 ml/kg of saline containing 2.7 \( \mu \)g of KH2PO4 radiolabelled phosphorus (NEN Lifescience Product, UK) was given to the rats by intravenous (i.v.) administration. At 15 and 90 min, peripheral blood samples were collected from the tail vein and the radioactivity in 100 \( \mu \)l samples was determined using a liquid scintillation counter (Packard Japan). The radioactive phosphate clearance rate (Jt) was determined using the following equation:

\[
Jt (\text{pmol/min}) = \frac{(C90\text{min} – C15\text{min})}{C \text{pmol}/75}
\]

where C90min and C15min were defined as radioactivity counts at 90 and 15 min, respectively. C pmol was defined as a radioactivity count of 1 pmol KH2PO4 radiolabelled phosphorus.

Preparation of brush border membrane and western blot analysis

BBMs were prepared from rat jejunum and kidney by the Ca precipitation method as previously described [10,16]. Purity of the membranes was assessed by measurement of alkaline phosphatase levels. Antibodies to NaPi-2b and NaPi-2a were generated against the C-terminal amino acid sequence as previously described [11]. Antibody to sodium-dependent glucose transporter (SGLT) was obtained from a commercial vendor (Chemicon International, Temecula, CA). A 20 \( \mu \)g aliquot of BBM protein was subjected to SDS–PAGE, and the resolved protein was transferred to a nylon membrane (Millipore Japan). NaPi-2b antibody and NaPi-2a hybridization was performed in solutions containing 1/1000 NaPi-2b antibody and 1/2000 NaPi-2a antibody, respectively. SGLT-1 hybridization was performed in solutions containing 1/1000 SGLT antibody.

Renal histology

At sacrifice, the right kidney was fixed in 10% formalin and embedded in paraffin. Sections (5 \( \mu \)m) were stained...
with haematoxylin–eosin (HE) and used to identify 2,8-dihydroxyadenine (DHA) crystals.

**Statistical analysis**

Results are expressed as means±SE. Comparisons between groups were evaluated by Student’s *t*-tests. A *P*-value of <0.05 was considered significant.

**Results**

**Effect of nicotinamide on serum chemistry**

There were no significant differences in average daily food intake during the study (vehicle treated, 8.52±1.26 g/day; nicotinamide treated, 8.92±0.85 g/day), or in body weight between the nicotinamide- and vehicle-treated groups at the end of the study (Table 1). An increase in serum Pi levels was seen from day 0 in rats fed the adenine-containing diet (adenine diet, 11.46±0.37 mg/dl; normal diet control, 8.33±0.23 mg/dl), and further increases were observed as renal insufficiency progressed. By the end of the experiment, however, this gradual increase in serum Pi caused by renal insufficiency was almost completely blocked by the administration of nicotinamide (Figure 1A). A decrease in serum Ca level was observed from day 4 in the vehicle-treated but not in the nicotinamide-treated group (Figure 1B). The serum calcium–phosphorus product was significantly decreased on day 6 in rats treated with nicotinamide (Figure 1C). There were no significant differences in either serum 1,25(OH)2D levels or glucose levels between the two groups at the end of the study (Table 1).

Because serum phosphate levels are mainly regulated by the renal phosphate re-absorption rate, we next examined fractional excretion of phosphate in the vehicle- and nicotinamide-treated groups using urine samples collected during the last 24 h of the experiment. As shown in Table 1, the fractional excretion of phosphate (FEPi) was not affected by treatment with nicotinamide. Changes in the expression of NaPi-2a on the apical membranes of proximal tubular epithelial cells are known to be the most important determinants of renal phosphate re-absorption in proximal tubules. From western blot analysis, NaPi-2a protein could not be detected in BBMs from the vehicle- and nicotinamide-treated group, which is in contrast to the control group (Figure 2A).

These findings indicate that the antihyperphosphataemic effect of nicotinamide in these rats was attributable to extrarenal phosphate handling.

**Effect of nicotinamide on intestinal Pi absorption**

As shown in Figure 3, peripheral blood Pi at 45 min after the administration of radiolabelled phosphate on day 6 was significantly lower in the nicotinamide-treated group than in the vehicle-treated group.

**Effect of nicotinamide on blood Pi clearance**

The blood Pi clearance rate (*Jt*), which was calculated by assessing the peripheral blood radioactivity count at 15 and 90 min after i.v. administration of radioactive phosphate, did not differ between the two groups, suggesting that there was no difference in their renal excretion or the tissue distribution of phosphate (vehicle, 1.59±0.07 pmol/min; nicotinamide treated, 1.34±0.19 pmol/min, NS).

Taken together, these data indicate that nicotinamide treatment suppressed intestinal Pi absorption but not Pi excretion or distribution in vivo.

**Western blot analysis**

Compared with the vehicle-treated group, the nicotinamide-treated group exhibited a marked decrease in NaPi-2b expression in jejunum BBMs (Figure 2B). This decrease appeared to be regulated in a specific manner, since there was no effect on the expression of SGLT-1 (Figure 2C).

**Table 1. Development of adenine-induced chronic renal failure and treatment with nicotinamide**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Adenine-induced CRF</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>No. of rats</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>227.36±6.10</td>
<td>196.43±6.35*</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>17.13±0.61</td>
<td>8.52±1.26*</td>
</tr>
<tr>
<td>FEPi (%)</td>
<td>–</td>
<td>28.2±2.9</td>
</tr>
<tr>
<td>Urinary Pi (mg)</td>
<td>82.82±9.49</td>
<td>80.77±6.35</td>
</tr>
<tr>
<td>Serum 1,25(OH)2D (pg/ml)</td>
<td>327.48±57.52</td>
<td>64.75±21.28**</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>162.50±4.11</td>
<td>116.45±2.55**</td>
</tr>
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A summary of the serum and urinary scores at the end of the study is shown. Values are means±SE (n=10).

*P<0.01, **P<0.001 vs sham-treated rats that received vehicle treatment at the end of the study.

**Effect of the nicotinamide treatment on renal function**

BUN and serum creatinine levels sharply increased in the vehicle-treated group during the last 6 days, indicating a progression of renal insufficiency during this period. Nicotinamide significantly attenuated the increase in both serum creatinine (Figure 4A) and serum BUN (Figure 4B) levels. Furthermore, creatinine clearance at the end of the study was maintained at a significantly higher level in the nicotinamide-treated group than in the vehicle-treated group (Figure 4C). Final kidney weights did not differ significantly between the groups (vehicle, 3.39±0.1 g; nicotinamide treated, 3.77±0.27 g, *P=0.084*).
Discussion

This study demonstrated that nicotinamide exerted an anti-hyperphosphataemic effect in rats with dietary adenine-induced chronic renal insufficiency. Treatment with nicotinamide for 6 days did not affect food consumption, body weight or serum glucose levels in these rats, indicating that the treatment did not induce adverse nutritional effects during the study. The observed anti-hyperphosphataemic effect appeared to be identical to the hypophosphataemic action of nicotinamide documented in a previous report with normal rats [10]. This previous study, however, did not investigate renal phosphate excretion regulation [10]. We demonstrated that the effect of nicotinamide was probably not mediated by renal phosphate regulation, since the fractional excretion of phosphate (FEPi) and the NaPi-2a protein content in renal BBMs from vehicle- and nicotinamide-treated groups were closely similar. Our results mirror clinical findings, in which the administration of niceritorol, another derivative of nicotinic acid, was shown to reduce serum phosphate levels without changing renal phosphate excretion [7].

To investigate extrarenal modification of phosphate regulation by nicotinamide, we next examined whether it affects the intestinal transport of radiolabelled phosphate. Our results showed that nicotinamide decreased the transport of orally administered phosphate into the blood. Previous studies have shown that nicotinamide decreases phosphate transport in BBM vesicles [10] and increases its fecal elimination in normal rats [9]. To our knowledge, the present results are the first to demonstrate that nicotinamide decreases intestinal phosphate absorption \textit{in vivo}.

Because this decrease in absorption may represent the net effect of not only intestinal Pi absorption but also tissue distribution and renal excretion, which may affect Pi levels even when intestinal absorption remains unchanged, we also investigated the blood Pi clearance \((J_t)\) of radiolabelled phosphate by using

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

\textbf{Fig. 1.} Effect of nicotinamide on levels of serum phosphate (A), calcium (B) and the calcium \(\times\) phosphate product (C) in rats with CRF. Values are means \pm SE \((n = 10)\). Rats were injected with vehicle (closed circle) or nicotinamide (closed square) for 6 days. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) vs rats receiving vehicle.
i.v. administration. Clearance values, including those at the 45 min sampling point used in the peroral administration experiment, did not differ between the groups, suggesting that tissue distribution and renal excretion of Pi did not affect the present findings.

It was proposed recently that NaPi-2b plays an important role in intestinal Pi absorption. In the present study, we demonstrated that nicotinamide treatment attenuated intestinal NaPi-2b expression. This downregulation of NaPi-2b appeared to be a

Fig. 2. Effect of nicotinamide on the expression of NaPi-2a, NaPi-2b and SGLT-1. NaPi-2a protein amounts in 20 μg of renal brush border membrane (A) and NaPi-2b (B) or SGLT-1 protein (C) in 20 μg of jejunum brush border membrane were analysed by western blot analysis.

Fig. 3. Effect of nicotinamide on the intestinal absorption of phosphate. Rats were injected with vehicle or nicotinamide for 6 days. Values are means ± SE (n = 6). *P < 0.05 vs rats with vehicle treatment.

Fig. 4. Effect of nicotinamide on levels of serum creatinine (A) and BUN (B) in rats with CRF. Values are means ± SE (n = 10). Rats were injected with vehicle (filled circle) or nicotinamide (filled square) for 6 days. *P < 0.05, **P < 0.01, ***P < 0.001 vs rats with vehicle treatment. (C) Creatine clearance at the end of the study. Values are means ± SE (n = 10). Rats were injected with vehicle (open column) or nicotinamide (filled column) for 6 days. *P < 0.05 vs rats with vehicle treatment.

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It was proposed recently that NaPi-2b plays an important role in intestinal Pi absorption. In the present study, we demonstrated that nicotinamide treatment attenuated intestinal NaPi-2b expression. This downregulation of NaPi-2b appeared to be a
specific effect of nicotinamide, since there was no effect on other BBM transporters, such as SGLT-1. In a previous study, nicotinamide decreased sodium-dependent phosphate uptake in rat intestinal BBM vesicles without causing significant changes in Pit-1 and Pit-2, two other Na/Pi co-transporters [10]. The present results therefore suggest that the decrease in intestinal Pi absorption by nicotinamide is partly due to a decrease in NaPi-2b expression on jejunal BBMs, and that NaPi-2b plays an important role in intestinal Pi absorption.

It has been reported that the expression of intestinal NaPi-2b is downregulated by a high Pi diet which leads to an increase in serum Pi, whereas it is upregulated by a low Pi diet, leading to a decrease in serum Pi [13]. Furthermore, an increase in serum 1,25(OH)\textsubscript{2}D levels results in an upregulation of NaPi-2b expression [13–15]. Compared with control rats in the present study, the adenine-induced CRF rats had frankly elevated serum Pi levels and reduced levels of serum 1,25(OH)\textsubscript{2}D. We therefore predicted that the expression of NaPi-2b would be decreased under these conditions. We found, however, that levels were maintained at close to normal. This finding suggests that the regulation of NaPi-2b expression may be modified in CRF. However, nicotinamide decreased the expression of NaPi-2b in these rats, suggesting that the downregulation is directly induced by nicotinamide.

In our study, the progression of renal insufficiency, which was characterized by increases in BUN and serum creatinine levels, appeared to be prevented by treatment with nicotinamide. Creatinine clearance was also improved by treatment with nicotinamide. Dietary Pi restriction has also been reported to prevent a deterioration in renal function [3,4,17], and phosphate binders that capture phosphate in the intestine have been shown to have protective effects against the progression of renal insufficiency when administered to animal models of CRF [6,18]. These findings indicate that the prevention of hyperphosphataemia by decreasing intestinal phosphate absorption is protective against the progression of renal insufficiency.

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It remains possible, however, that the protective effects of nicotinamide on renal function may be independent from effects on phosphate. Two other mechanisms are possible. First, nicotinic acid and nicotinamide are some of the smallest biologically active pyridine derivatives of vitamin B3, and are the major precursors to nicotinamide adenine dinucleotide (NAD). Kempson et al. [19] reported that nicotinamide treatment stimulated the production of NAD in renal proximal tubular cells. Since an increase in nicotinamide concentration leads to an increase in NAD molecules that are able to take part in energy metabolism, renal metabolism and its function may be directly improved by nicotinamide. In addition,
since NAD is reported to be an NaPi inhibitor in the kidney [19], elevated NAD production may directly inhibit intestinal NaPi-2b activity.

As a second possible mechanism for the protective effects on renal function, nicotinamide may inhibit the progression of renal failure by preventing DHA deposition in the kidney, especially since DHA deposition caused CRF in a model of renal failure similar to that used in the present study [20]. The staining, however, revealed no significant differences in kidney DHA crystal deposition between the groups (Figure 5). Moreover, there were no differences in urine volume or kidney size at the end of the experiment. These results indicate that DHA deposition had no influence on the present set of findings.

In conclusion, nicotinamide prevented increases in serum Pi in a rat model of CRF by decreasing intestinal phosphate absorption. Downregulation of NaPi-2b appeared to be an important mechanism for this effect. Nicotinamide also showed protective effects against the deterioration of renal function in these rats. This effect was possibly mediated by the inhibition of intestinal phosphate absorption, although other possibilities cannot be ruled out. Established clinical benefits of inhibiting phosphate absorption in CRF and dialysis patients include protection against the progression of renal insufficiency, the development of secondary hyperparathyroidism, and ectopic calcification. The present findings of nicotinamide-induced changes in intestinal phosphate absorption, especially in the regulation of NaPi-2h, may lead to a more practical approach for decreasing intestinal phosphate absorption, in terms of both compliance and safety, than the methods currently in use.

Conflict of interest statement. None declared.

References


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