Upregulation of Intestinal NaP_i-IIb Co-Transporter Gene Expression by Neuropeptide Y in Human Intestinal Caco-2 Cells

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Abstract: The aim of the present study is to examine the effect of neuropeptide Y and estrogen on type IIb sodium-coupled phosphate co-transporter (NaP_i-IIb) gene expression and activity. Uptake studies with human intestinal cell line, Caco-2 cells, showed that treatment with neuropeptide Y and estrogen concentration-dependently increased sodium-dependent phosphate absorption. Reverse transcriptase-polymerase chain reaction analysis indicated that expression of NaP_i-IIb mRNA was also increased after Y treatment with neuropeptide and estrogen. These studies show for the first time that neuropeptide Y increased intestinal sodium-dependent phosphate absorption. This increase is associated with the increase of the expression of NaP_i-IIb mRNA.

Keywords: Type IIb sodium-phosphate co-transporter, SLC34A2, Caco-2 cells, Neuropeptide Y, Estrogen.

INTRODUCTION

Intestinal phosphate (P_i) absorption across the apical membrane of small intestinal epithelial cells is mediated by the type IIb Na-coupled phosphate co-transporter (NaP_i-IIb). The cDNA encoding this transporter has been cloned from several species, including human [1, 2], rat [3] and mouse [4]. Intestinal P_i absorption through the NaP_i-IIb is regulated by various physiological effectors. Epidermal growth factor [5] and glucocorticoids [6] inhibit intestinal sodium-dependent P_i absorption and NaP_i-IIb gene expression, whereas dietary P_i deprivation stimulates intestinal sodium-dependent P_i absorption and NaP_i-IIb gene expression [7]. Previously, vitamin D had been considered as an essential factor for adaptation of intestinal NaP_i IIb to dietary P_i deficiency. However, Segawa *et al.* [8] recently demonstrated that intestinal NaP_i-IIb adaptation to a low-P_i diet occurs independently of vitamin D using vitamin D receptor null mice. The critical factor(s) for upregulation of intestinal NaP_i IIb for adaptation to a low-P_i diet have not been clarified yet.

Renal P_i reabsorption is mediated by the type IIa and type IIc sodium-coupled phosphate co-transporters located in the apical membrane of the proximal tubule [1, 4, 9, 10]. Recently, Mulroney *et al.* [11] reported that NaP_i-II-like transporters in the brain are regulated by both dietary P_i and cerebrospinal fluid P_i concentrations, and that the central P_i milieu can regulate renal NaP_i-2 expression. This report implies that a factor derived from central nervous system may help regulate whole body P_i homeostasis.

Neuropeptide Y is a potent orexigenic peptide that is implicated in the feeding response to a variety of stimuli [12]. Neuropeptide Y is known to modulate both central and peripheral control of gastrointestinal motility [13,14], and is found in sympathetic nerve fibers together with norepinephrine [15]. Subcellular fragmentation experiments suggest that neuropeptide Y and norepinephrine are colocalized in large dense-core vesicles [16]. In addition, neuropeptide Y gene expression, storage, and release are all increased after sympathetic nerve activation such as seen during hypoglycemia [17]. In the guinea-pig gut, neuropeptide Y immunoreactivity is found in 25% of all submucosal neurons, many of which innervate the intestinal mucosa and have been traced to epithelial cells [18, 19]. These facts raised the possibility that neuropeptide Y may be a factor which regulates Na-dependent P_i uptake in intestine. However, there is no evidence to date demonstrating regulation of NaP_i-IIb expression *via* neuropeptide Y. Previous results have shown that Caco-2 cells endogenously express the NaP_i-IIb gene [5]. Thus, the current studies were designed to investigate the possible link between neuropeptide Y and intestinal NaP_i-IIb expression using Caco-2 cells. To determine the reasonability of the experimental system in the present study, the effect of estrogen, which is known to upregulate NaP_i-IIb expression in the rat [20], was also tested.

MATERIAL AND METHODS

Cell Culture

Human intestinal epithelial Caco-2 cells were grown as a monolayer in Dulbecco's-modified Eagle medium with 10% fetal bovine serum, 1% GlutaMax (Invitrogen, Carlsbad, CA) and 1% HyQ-MEM (HyClone, Logan, UT). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. When cells became confluent, the medium was aspirated and changed to HEPES-buffered Krebs buffer comprised of (in mM) 137 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 Glucose, 10 HEPES, pH 7.0 adjusted with NaOH, and 0.1 - 10 nM neuropeptide Y

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(Peptide Institute, Osaka, Japan), 10 - 1000 nM β-estradiol (Sigma, St. Louis, MO) or vehicle (phosphate buffer saline) was added 24 h before the start of experiments.

Phosphate Uptake Studies with Intact Cells

Cells grown in 12-well plates with or without treatment with the drug were washed 3 times with phosphate uptake buffer comprised of (in mM) 137 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 10 HEPES, pH 7.0 adjusted with Tris base, and afterwards incubated with 1 ml of the same buffer with 500 μM KH_2PO_4 and [^{32}P] KH_2PO_4 (1 μCi/ml). After 15 min, the uptake buffer was removed and the cells were washed 3 times with ice-cold stop buffer comprised of (in mM) 137 NaCl, 10 HEPES, pH 7.0 adjusted with Tris base, and lysed with 2N NaOH. The lysate was used for scintillation counting and determination of protein concentration according to Lowry *et al.* [21]. To determine the sodium-independent P_i transport, NaCl in the uptake buffer was replaced by choline chloride in some experiments.

Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis of NaP_i-IIb Gene Expression in Caco-2 Cells

Total RNA was purified from Caco-2 cells using the Trizol reagent (Invitrogen), and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) for 60 min at 42°C primed with oligo$(DT)_{12\text{-}18}$ primer (Invitrogen) at conditions recommended by the manufacturer. Polymerase chain reaction (PCR) was performed with ExTaq DNA polymerase (Takara Bio, Otsu, Japan) using a programmable thermal cycler (PTC-100, MJ Research, Waltham, MA). Primers for amplication of human NaP_i IIb (sense 5'-TGCTCTTGCCCGTGGAGGTG-3'; antisense 5'-CCTCA ATGCATTGCCAGGGCT-3') were purchased from Nihon Gene Research Laboratories (Sendai, Japan). As an internal control, β-actin DNA was amplified using its specific primers (sense 5'-GTGGGCCGCCCTAGGCACCA-3'; antisense 5'-TTAATGTCACGCACGATTTC-3'; Nihon Gene Research Laboratories). The PCR conditions were as following; 34 cycles of 94°C for 30 s, 65°C for 30 s and 68°C for 1 min for NaP_i IIb, and 34 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 1 min for β-actin. Subsaturation levels of cDNA templates that were needed to produce a dose-dependent amount of PCR product were defined in pilot experiments by testing a range of template concentrations. Subsequent PCR was carried out with subsaturation levels of RT reactions with identical amplification parameters. PCR was performed with human NaP_i-IIb or β-actin primers in separate reactions; equal volumes (10 μl) of the PCR products were separated on a TBE - 1.5% agarose gel and stained with 0.1% ethidium bromide. The density of the bands was analyzed with Image J (National Institute of Health, Bethesda, MD). NaP_i-IIb mRNA expression levels were estimated by taking a ratio of NaP_i-IIb to β-actin amplicon optical densities.

Statistical Analysis

Each datum is expressed as a mean ± S.E.M. One way analysis of variance followed by Bonferroni's post-hoc test was used for multiple comparisons. Differences were considered to be statistically significant when the *P* value was less than 0.05.

RESULTS AND DISCUSSION

To determine the reasonability of the experimental system in the present study, we tested the effect of estrogen, which is known to upregulate NaP_i-IIb expression in the rat [20]. NaP_i-IIb gene expression was concentration-dependently increased in the β-estradiol-treated Caco-2 cells (Fig. **1A**). The mRNA levels in 100 nM β-estradiol-treated cells are about 1.6-fold relative to the control cells and this result is quite consistent with that in a previous report [20]. That in 1 μM β-estradiol-treated cells is about 2-fold. Na-dependent P_i absorption was also measured in β-estradiol-treated Caco-2 cells. Treatment with β-estradiol induced a significant increase of Na-dependent P_i absorption, whereas Na-independent P_i absorption was not affected (Fig. **1B**). Compared with vehicle-treated cells, Na-dependent P_i uptake increased about 20% in 1 μM β-estradiol-treated cells. This result is consistent with that found on intestinal brush-border membrane vesicles from β-estradiol-treated rats [20]. Based on these observations, the experimental system used in the present study is suitable for determination of the factors for regulation of intestinal NaP_i-IIb gene expression and activity.

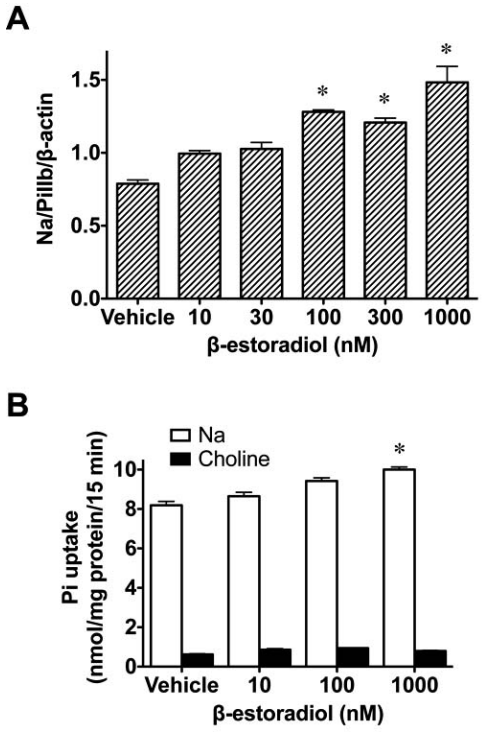

Fig. (1). Effect of β-estradiol on NaP_i-IIb mRNA levels and activity in human intestinal epithelial (Caco-2) cells. Caco-2 cells were grown in vehicle-containing medium or β-estradiol-containing HEPES-buffered Krebs buffer (10 - 1000 nM) for 24 h. Total RNA was then isolated from these cells and used for cDNA synthesis. Subsequent PCR was performed with human NaP_i-IIb or β-actin primers in separate reactions. Equal volumes of NaP_i-IIb and β-actin PCR reactions were then loaded on the agarose gel and visualized with ethidium bromide. (**A**) analysis of optical density of gel images. (**B**) Sodium-dependent (open column) and -independent (closed column) phosphate uptake in Caco-2 cells. Phosphate uptake was measured in the presence of 500 μM KH_2PO_4 for 15 min. Each datum is presented as a ratio of NaP_i-IIb to β-actin band intensities, and expressed as mean ± S.E.M. of 3 separate experiments. **P*<0.05 *vs* the vehicle-treated group.

NaP_i-IIb gene expression was concentration-dependently increased in the neuropeptide Y-treated Caco-2 cells (Fig. **2A**). The mRNA levels in 1 nM neuropeptide Y-treated cells are about 1.7-fold relative to the control cells. Na-dependent P_i absorption was also measured in neuropeptide Y-treated Caco-2 cells. Treatment with neuropeptide Y induced a significant increase of Na-dependent P_i absorption, whereas Na-independent P_i absorption was not affected (Fig. **2B**). Compared with the vehicle-treated cells, Na-dependent P_i uptake for 15 min increased about 20% in 1 nM neuropeptide Y-treated cells. These results suggest that neuropeptide Y increases both the expression of NaP_i-IIb mRNA and the activity of the co-transporter. The increase in Pi uptake is smaller than that in mRNA level of NaPi-IIb in the present study. Although the underlying mechanism is known, there are some possibilities; for example, (1) the protein level of NaPi-IIb may not be increased in proportion to mRNA level, (2) the measuring time of Pi uptake (for 15 min) is too long. In the previous report [20], β-estradiol induced a significant increase of Pi uptake when the measuring time is for 10 s, but did not show any increase when the measuring time is for 3 h. The increase in the number of the transporter will accelerate an initial rate of the uptake. When a measuring time is too long, the uptake will be saturated. It is possible that the measuring time in the present study is a little long to detect the difference of the initial uptake rate.

In the present study, neuropeptide Y showed similar results as estrogen, which is shown to increase NaPi-IIb expression *in vivo* [20]. Therefore, the results in the present study may suggest a physiological role of neuropeptide Y in the regulation of NaPi-IIb expression. However, the mechanism of the effect of neuropeptide Y is still unclear, because there are few commercially available ligands. According to Sgambati *et al*. [22], Caco-2 cells express Y2 receptors. Therefore, Y2 receptor may be involved in the effects seen in the present study. Although there is no information about an acute direct activation of NaPi-IIb by neuropeptide Y, the possibility that the acute activation is involved in the increase of Pi uptake could not be excluded by the present study. Further studies are needed to confirm physiological relevance in animal models and the mechanism of the effects of neuropeptide Y.

Neuropeptide Y is known to be in sympathetic nerve fibers with norepinephrine [15], and thought to be involved in the feeding response to a variety of stimuli [12]. Many neuropeptide Y neurons innervate the intestinal mucosa in the guinea-pig and have been traced to epithelial cells [18, 19]. The expression, storage and release of neuropeptide Y are all increased after sympathetic nerve is activated, for example during hypoglycemia [17]. From these facts, we hypothecated that neuropeptide Y released in the fasting period is possible to upregulate Na-dependent P_i uptake to improve P_i absorption in intestine.

Although the present study showed that neuropeptide Y increased intestinal sodium-dependent phosphate absorption, the specificity of the effect has been still unknown. It is possible that the effects of neuropeptide Y on other solutes uptakes influence the rate of uptake of phosphate. Further studies are needed to elucidate the specificity of the described effects of neuropeptide Y.

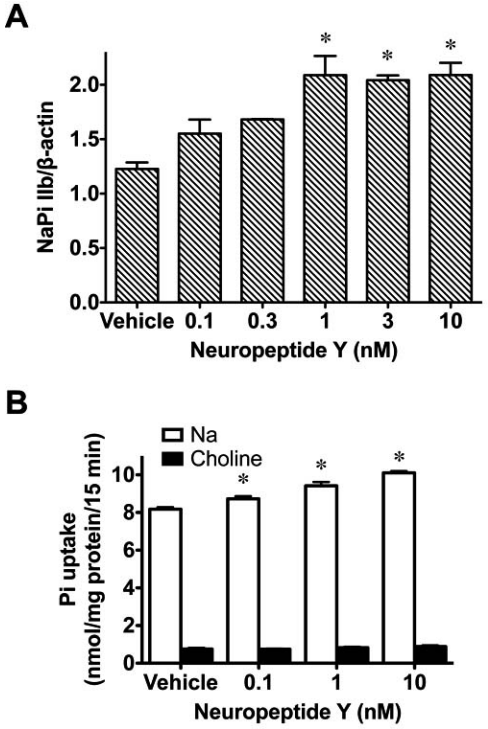

Fig. (2). Effect of neuropeptide Y on NaP_i-IIb mRNA levels and activity in human intestinal epithelial (Caco-2) cells. Caco-2 cells were grown in vehicle-containing medium or neuropeptide Y-containing HEPES-buffered Krebs buffer (0.1 - 10 nM) for 24 h. Total RNA was then isolated from these cells and used for cDNA synthesis. Subsequent PCR was performed with human NaP_i-IIb or β-actin primers in separate reactions. Equal volumes of NaP_i-IIb and β-actin PCR reactions were then loaded on the agarose gel and visualized with ethidium bromide. (**A**) analysis of optical density of gel images. (**B**) Sodium-dependent (open column) and -independent (closed column) phosphate uptake in Caco-2 cells. Phosphate uptake was measured in the presence of 500 μM KH_2PO_4 for 15 min. Each datum is presented as a ratio of NaPi-IIb to β-actin band intensities, and expressed as mean ± S.E.M. of 3 separate experiments. **P*<0.05 *vs* the vehicle-treated group.

We used human intestinal epithelial cell line, Caco-2 cells, as an *in vitro* model of intestine. Our results demonstrate that endogenous NaP_i-IIb gene expression was stimulated by estrogen treatment in Caco-2 cells and the increase in NaP_i-IIb mRNA abundance in Caco-2 cells after estrogen treatment is similar to the increase reported previously [20], suggesting that the Caco-2 model used in the present study is very useful for investigating the mechanism regulating NaP_i-IIb activity in intestine.

In summary, we show that neuropeptide Y increases intestinal Na-dependent P_i uptake in Caco-2 cells at least in part through an increase of NaP_i-IIb mRNA levels.

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