Regulation of intestinal NaPi-IIb cotransporter gene expression by estrogen

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Estrogen is an important physiological regulator involved in modulating calcium homeostasis (14, 21). Estrogen is involved not only in the regulation of Ca2+ absorption (3, 13) but also in the maintenance of bone density (3, 8) and in the modulation of 1,25(OH)2 vitamin D3 synthesis (4, 8, 20, 23, 27). Although 1,25(OH)2 vitamin D3 is known to regulate intestinal Pi absorption, there is no evidence to date demonstrating estrogen regulation via NaPi-IIb cotransporter expression. Thus the current studies were designed to investigate the possible link between estrogen and intestinal NaPi-IIb gene expression.

As reported in the current communication, we initially detected a significant increase in brush-border membrane Na/Pi uptake and NaPi-IIb mRNA abundance in estrogen-treated female rats. These results suggested a possible role for estrogen in NaPi-IIb gene regulation. Therefore, to further understand the role of estrogen in intestinal Pi absorption, we developed a rat NaPi-IIb antibody, characterized expression of NaPi-IIb protein and mRNA in estrogen-treated rats, and tested the response of the human NaPi-IIb gene promoter to estrogen in Caco-2 cells. These are the first studies that exemplify direct regulation by estrogen of intestinal phosphate absorption as likely mediated by the NaPi-IIb cotransporter.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (95–100 days of age) were used for these studies. Animals received subcutaneous injections of β-estradiol (2 mg/kg body wt, one dose; Sigma, St. Louis, MO) or vehicle [ethanol (1 dose)/propylene glycol (4 doses), vol/vol] alone. After the injection (16 h), rats were killed, and the jejunal mucosa was harvested and used for mRNA and brush-border membrane vesicle (BBMV) purification. All animal work has been approved by the University of Arizona Institutional Animal Care and Use Committee. All experiments were repeated at least three times with different groups of animals (3–4 rats/group).

Cell culture. Human intestinal epithelial (Caco-2) cells were purchased from American Type Culture Collection (ATCC) and cultured according to ATCC guidelines. Cells were cultured at 37°C in a 95% air-5% CO2 atmosphere and passaged every 72 h. In some experiments, cells were incubated with 0.1 μM estradiol for 24 h. Prior to experiments, cells were passaged every 72 h. In some experiments, cells were incubated with 0.1 μM estradiol for 24 h. Prior to experiments, cells were incubated with 0.1 μM estradiol for 24 h.

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bated with 100 nM β-estradiol or vehicle (ethanol) for 16 h before harvest. For transcription studies, cells were pretreated with actinomycin D (100 nM; Calbiochem-Novabiochem, San Diego, CA) for 2 h and then were treated with 100 nM β-estradiol for 16 h in the presence of actinomycin D before harvesting cells. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA).

Phosphate and glutamine uptake analysis in BBMV. BBMVs were prepared from rat jejunal mucosa. Pi and glutamine uptake were measured as previously described (1, 6). The contribution of sodium-dependent uptake was calculated by subtracting the sodium-independent uptake values observed in the absence of sodium from the uptake values in the presence of sodium. Sodium-dependent glutamine uptake measurements were performed to determine whether the changes in Pi transport were specific to Na-Pi cotransport. This experiment was repeated with BBMV prepared from three to four different groups of animals.

RNA purification and Northern blot analyses. mRNA was isolated from rat jejunal mucosa using the Fast-Track mRNA purification kit (Invitrogen, Carlsbad, CA). mRNA (10 μg) was used for Northern blot analyses with rat NaPi-IIb cDNA probes (31) under high-stringency washing conditions, as described previously (9). 18S (encoding rat cyclophilin; see Ref. 10) cDNA specific probes were used as internal standards for quantitating NaPi-IIb gene expression. Blots were exposed to a phosphorimaging screen, and band intensities were determined with Quantity One Software (FX Molecular Imager; Bio-Rad, Hercules, CA). NaPi-IIb gene expression levels were estimated by taking the ratio of hybridization intensities of NaPi-IIb mRNA over 18S mRNA. The experiment was repeated with mRNA isolated from three different groups of animals.

Production of NaPi-IIb antiserum. Rabbit polyclonal antibodies were raised against synthetic peptides (MAPW-PELENAQPNPGKFIEGA) designed from the NH2-terminus of the mouse NaPi-IIb protein (Research Genetics, Huntsville, AL). This peptide has high homology with the rat NaPi-IIb protein. The peptides were conjugated to keyhole limpet hemocyanin and injected into New Zealand White rabbits. The rabbits received multiple immunogenic boosts over a several-week period and were bled periodically throughout this process. The anti-peptide antibody titer was determined by an ELISA and used to measure the antigenic properties of the animal bleeds. All bleeds had an ELISA of >204,800, which is the upper threshold of the assay. All serum was separated into aliquots and stored at −20°C.

PCR analysis to detect NaPi-IIb expression in Caco-2 cells. mRNA was purified from Caco-2 cells using the Fast-Track mRNA purification kit (Invitrogen). RT-PCR conditions were identical to those described previously (31). The primers used to detect NaPi-IIb were designed from the human NaPi-IIb cDNA sequence (GenBank accession no. AF146796). The forward primer was at 1264–1283 bp (5′-TTGCATGTTTGACTGGCTAC-3′), and the reverse primer was at 1,795–1,814 bp (5′-CGGCAGGAAGTCCAGTTCT-3′). The expected amplicon size from NaPi-IIb mRNA is 550 bp. The primers used to detect β-actin were purchased from Stratagene (La Jolla, CA). The size of the amplified product from the β-actin transcript is 661 bp.

Western blot analysis. Protein (40 μg) was loaded on 10% SDS-PAGE gels. Proteins were then blotted on a nylon membrane for immunoblot analysis. A 1:4,000 dilution of the primary or preimmune serum was used in these experiments. For blocking experiments, the primary antiserum was incubated with the antigenic peptide at a concentration of 1 mg/ml for 1 h at 15°C before use. A 1:5,000 dilution of the β-actin antiserum (Sigma) was used to detect β-actin protein abundance. Western detection was performed with the BM chemiluminescence Western blotting kit (mouse/rabbit; Roche Diagnostics, Mannheim, Germany). Protein expression level quantitation, a ratio of NaPi-IIb protein intensity over β-actin protein intensity was used. Western blotting experiments on rat intestine were repeated three times with tissue isolated from three different groups of animals.

Immunohistochemistry. Rat jejunal intestine was removed, flushed, and cut into 2-mm transverse sections. Rat lungs were removed en masse and inflated with Streek Tissue Fixative (Streek Laboratories, Omaha, NE) at 20 cm pressure for 1–2 h. The inflated lungs were left in fixative for 2–3 h after the trachea was tied closed and then transferred to a
30% sucrose-PBS solution at 4°C overnight. Thin slices of tissues were cut and embedded in paraffin. Tissue sections (6–10 μm) from both tissues were prepared by the Histopathology Core Service at the University of Arizona (Tucson, AZ). Immunohistochemical staining and detection was performed as previously described (2). NaPi-IIb antiserum was reacted with sections for 60 min at a 1:500 (for intestinal sections) or a 1:4,000 (for lung sections) dilution in PBS. Some sections were reacted with preimmune serum at the same dilution. The small intestinal slides were subsequently reacted with secondary antiserum (Alexa Fluor 647 goat anti-rabbit IgG; Molecular Probes, Eugene, OR) at a 1:500 dilution and visualized by confocal microscopy MRC-1024ES laser scanning confocal (Bio-Rad) equipped with a Nikon TE-300 research grade microscope] using the HQ-598–40 emission filter and an excitation wavelength of 568 nm. For lung sections, the secondary antibody (Alexa Fluor 647 goat anti-rabbit IgG; Molecular Probes) was used at 5 μg/ml for 1 h, and staining was visualized with the 647-nm laser line and the 680 DF 32 Emission Filter. All images from each tissue were captured with identical settings on the laser scanning confocal microscope.

**Statistical analysis.** ANOVA post hoc tests (StatView version 5.0.1; SAS Institute, Cary, NC) were used to compare values of the experimental data. *P* values <0.05 were considered significant.

**RESULTS**

Characterization of NaPi-IIb antibodies. Small intestinal BBM protein or lung membrane protein from rats (40 μg) was loaded on SDS-PAGE gels and blotted on nitrocellulose membranes for antibody characterization studies by immunoblot analysis. Mouse intestinal BBM protein and lung protein were also used to test the antiserum. As shown in Fig. 1, a single immunoreactive band at ~80 kDa was detected with NaPi-IIb antiserum in rat intestinal and lung tissues. This immunoreactive band was blockable by the antigenic peptide and was absent when the preimmune serum was used (Fig. 1A). Furthermore, in mouse lung and intestinal tissues, this antiserum recognized ~80 kDa and ~110 kDa protein bands (Fig. 1B). This finding is consistent with previously published observations from our laboratory that showed specific recognition of two immunoreactive bands of similar size in mouse intestine with a COOH-terminal NaPi-IIb peptide antiserum (2). Immunohistochemical analysis of rat intestine and lung using this NH2-terminal antibody showed specific recognition of the NaPi-IIb protein only on the apical membrane of the intestinal epithelium (Fig. 2A) and on the apical membranes of type II alveolar cells with the pulmonary epithelium (Fig. 2C). The preimmune serum did not react with any proteins in the rat intestine (Fig. 2B) or lung (Fig. 2D).

**Effect of β-estradiol treatment on BBMV phosphate and glutamine absorption in rat jejunum.** Adult female rats were treated with β-estradiol or vehicle. BBMV were purified from jejunal mucosa, and Na/Pi or Na/glutamine absorption was measured by a membrane filtration method. Results showed that β-estradiol treatment increased Na/Pi absorption (66 ± 2 nmol·mg protein−1·10 s−1 for control vs. 96 ± 8 nmol·mg protein−1·10 s−1 for β-estradiol treated; *n* = 6; *P* < 0.04).

Compared with vehicle-treated animals, Na/Pi uptake increased ~45% in β-estradiol-treated animals. To determine whether the effect of β-estradiol on Pi uptake is specific, sodium-dependent glutamine uptake experiments were also performed. Results showed that sodium-dependent glutamine uptake was similar in both groups (15 ± 3 nmol·mg protein−1·10 s−1 for controls and 14 ± 2 for β-estradiol treatment; *n* = 3; Fig. 3A). These data indicated that β-estradiol did not affect sodium-dependent glutamine absorption in rat small intestine.

**Effect of β-estradiol treatment on BBMV size in rat jejunum.** To determine if β-estradiol alters BBMV size, we measured 3-h equilibrium values for both Pi and glutamine uptake. As shown in Fig. 3B, Na/Pi uptake at 3 h was not different between β-estradiol-treated rats and control rats (18 ± 5 nmol·mg protein−1·3 h−1 for control vs. 21 ± 7 nmol·mg protein−1·3 h−1 for β-estradiol treatment; *n* = 3). Sodium-dependent glutamine uptake at 3 h was 9 ± 1 and 8 ± 3 nmol·mg
protein h\(^{-1}\) for control and \(\beta\)-estradiol-treated rats, respectively \((n = 3)\). These results suggested that \(\beta\)-estradiol treatment did not alter BBMV size.

**Effect of \(\beta\)-estradiol treatment on BBM NaPi-IIb protein expression in rat jejunum.** Adult rats were treated with \(\beta\)-estradiol or vehicle. BBM proteins were purified from rat jejunum, and 40 \(\mu\)g protein were used for Western blot analysis. Results showed that \(\beta\)-estradiol treatment increased NaPi-IIb immunoreactive protein abundance (indicated by the ratio of optical densities of the NaPi-IIb band to that of the \(\beta\)-actin band) from 11.3 ± 0.1 in vehicle-treated animals to 19.8 ± 2.4 in \(\beta\)-estradiol-treated animals \((n = 3; P < 0.04; \text{Fig. 4})\). The increase was \(\sim 75\% \) in treated animals compared with control animals.

**Effect of \(\beta\)-estradiol treatment on NaPi-IIb mRNA expression in rat jejunum.** Adult rats were treated with \(\beta\)-estradiol or vehicle, mRNA was purified from jejunal mucosa, and Northern blot analyses were performed with \(32\text{P}\)-labeled rat NaPi-IIb and 1B15 cDNA specific probes. Hybridization patterns clearly showed that intestinal NaPi-IIb mRNA abundance significantly increased in \(\beta\)-estradiol treated rats, but no change was detected in 1B15 mRNA abundance. NaPi-IIb mRNA abundance (indicated by the ratio of the signal intensities of the NaPi-IIb band to that of the 1B15 band) was increased by \(\sim 50\% \) from 0.11 ± 0.02 in control animals to 0.16 ± 0.03 in treated animals \((n = 3; P < 0.04; \text{Fig. 5})\).

\(\beta\)-Estradiol treatment increases NaPi-IIb mRNA abundance in Caco-2 cells.** Previous results have shown that Caco-2 cells endogenously express the NaPi-IIb gene (31). NaPi-IIb mRNA expression levels in Caco-2 cells, after exposure to vehicle or \(\beta\)-estradiol, were assessed by semiquantitative RT-PCR using human NaPi-IIb and \(\beta\)-actin cDNA-specific primers. Results showed that NaPi-IIb gene expression (indicated by the ratio of the optical densities of the NaPi-IIb band to that of the \(\beta\)-actin band) was significantly increased in \(\beta\)-estradiol-treated Caco-2 cells \((1.65 ± 0.10)\) compared with vehicle-treated cells \((1.02 ± 0.03; n = 4; P < 0.01)\). The increase was \(\sim 1.6\)-fold and could be abolished by actinomycin D treatment (Fig. 6).

\(\beta\)-Estradiol treatment increases human NaPi-IIb gene promoter activity in Caco-2 cells.** To determine whether the human NaPi-IIb gene promoter responds to \(\beta\)-estradiol treatment, four promoter constructs (pGL3/~2783 bp, pGL3/~1103 bp, pGL3/~563 bp, and
pGL3(−181 bp) were transfected in Caco-2 cells by a previously described method (31). To test the effect of β-estradiol on human NaPi-IIb gene promoter activity, Caco-2 cells were first transfected with promoter constructs and then treated with 100 nM β-estradiol or vehicle for 16 h before measuring reporter gene expression. The internal control, Renilla luciferase driven by the

Fig. 3. Effect of β-estradiol on rat intestinal sodium-dependent phosphate and glutamine absorption. Sodium-dependent phosphate and glutamine uptake analysis of BBM vesicles (BBMVs) isolated from rat jejunal mucosa treated with vehicle (−E2) or β-estradiol (+E2). The sodium-dependent uptake component was obtained by subtracting uptake values in the presence of KCl from uptake values in the presence of NaCl. A: sodium-dependent uptake for Pi and glutamine at 10 s with BBMVs isolated from jejunal mucosa of rats treated with vehicle or β-estradiol. Results are means ± SE from 3 separate experiments. *P < 0.04 for vehicle treatment vs. β-estradiol treatment. B: sodium-dependent uptake of Pi and glutamine at 3 h with BBMVs isolated from rat jejunal mucosa treated with vehicle or β-estradiol. Results are means ± SE from 3 or 4 separate experiments.

Fig. 4. Effect of β-estradiol on rat NaPi-IIb transporter protein abundance. BBMVs were isolated from the jejunal mucosa of rats treated with vehicle or β-estradiol. BBM protein (40 μg) was loaded on SDS-PAGE gels, and immunoblots were performed. A 1:4,000 dilution of the mouse NaPi-IIb antibody was used for detection. The NaPi-IIb antiserum detected a protein band at ~80 kDa. The β-actin antibody detected a protein band at the expected ~40 kDa. A: typical immunoblot experiments. B: relative increase in rat NaPi-IIb protein from 3 independent experiments. *P < 0.04 for vehicle treatment vs. β-estradiol treatment.

Fig. 5. Effect of β-estradiol on rat intestinal NaPi-IIb mRNA expression. mRNA (10 μg) isolated from jejunal mucosa of rats treated with vehicle or β-estradiol was fractionated by gel electrophoresis, blotted, and hybridized with rat NaPi-IIb and 1B15 cDNA-specific probes. NaPi-IIb probes recognized a hybridization signal at ~4.4 kb, and 1B15 probes recognized a hybridization signal at ~1.0 kb. A: typical experiment. B: phosphorimage analysis of rat intestinal NaPi-IIb mRNA abundance in vehicle- or β-estradiol-treated rat intestine. Results are means ± SE from 3 separate experiments. *P < 0.04 for vehicle treatment vs. β-estradiol treatment.
and in rat lung type II alveolar cells. Furthermore, this NH2-terminal antiserum identified similar-size protein bands (~80 and ~110 kDa) in mouse lung and intestine as COOH-terminal antibodies did in previous studies (2, 18, 26). Previous work identified ~110 and ~80 kDa band from 14-day-old rats (2), whereas in the current studies, we detected only ~80 kDa protein in adult rats. This protein size difference may be because of the differential posttranslational modification between suckling and adult rats. A similar observation has been reported in mice, where an ~88-kDa NaPi-IIb protein band was seen in suckling mice and a ~110-kDa NaPi-IIb was seen in adult mice. This difference was likely because of protein glycosylation (2).

In the current study, estrogen treatment stimulated BBMV Na/Pi uptake, but not sodium-dependent glutamine uptake. Three-hour equilibrium values of the vesicle populations from control and estrogen-treated rats were similar, which indicated that estrogen treatment did not alter BBMV size. Together, these results suggested that the increase in BBMV Na/Pi uptake by estrogen is specific and further that the increase in Na-Pi cotransport was not the result of an increase in vesicle size.

Western blot analysis results showed that estrogen treatment increased NaPi-IIb protein abundance in rat intestine. The increase in BBMV Pi uptake detected after estrogen treatment is most likely related to increased apical NaPi-IIb protein expression, due to the fact that estrogen treatment increased both intestinal NaPi-IIb mRNA and protein levels to a similar extent. Further studies showed that estrogen treatment also increased NaPi-IIb protein levels in rats. Taken together, these results suggest that estrogen increases intestinal Pi absorption through its stimulatory effect on NaPi-IIb cotransporter gene expression.

Fig. 6. Effect of β-estradiol on NaPi-IIb mRNA levels in human intestinal epithelial (Caco-2) cells. Caco-2 cells were grown in vehicle-containing medium, β-estradiol-containing medium (+E2; 100 nM), actinomycin D-containing medium (+ActD; 100 nM), or ActD-alone medium. Equal volumes of NaPi-IIb and β-actin primers in separate reactions. Equal volumes of NaPi-IIb and β-actin PCR reactions were then loaded on the same gel and visualized with ethidium bromide. A: typical experiment. B: optical density analysis of RT-PCR results in Caco-2 cells. Data are presented as a ratio of NaPi-IIb to β-actin band intensities. Results are presented as means ± SE from 3 separate experiments. *P ≤ 0.01 for β-estradiol treatment vs. vehicle treatment, ActD treatment, and ActD/β-estradiol treatment.

CMV promoter, in transfected Caco-2 cells. Data showed that β-estradiol treatment increased human NaPi-IIb promoter activity ~36% only in promoter construct pGL3−1,103, pGL3−563, pGL3−2,783-transfected cells, but not in cells transfected with the other shorter NaPi-IIb promoter constructs (Fig. 7).

DISCUSSION

Earlier studies have shown the presence of estrogen receptors in the intestinal epithelium (22, 24, 28). Studies also indicated that estrogen treatment could increase phosphate uptake in rabbit kidney (15) and modulate vitamin D3 synthesis (4, 8, 20, 23, 27). These observations suggested that estrogen might play a role in intestinal phosphate absorption.

To further characterize estrogen regulation of the NaPi-IIb cotransporter, we developed an NaPi-IIb antibody that specifically recognizes the rodent NaPi-IIb protein. This antibody was raised against a peptide from the NH2-terminal 21 amino acids of the mouse NaPi-IIb cotransporter. This region has high homology with the NH2-terminus of the rat NaPi-IIb protein sequence. This antibody could recognize the NaPi-IIb protein from rat intestine and lung at ~80 kDa and an in vivo-translated mouse NaPi-IIb protein (data not shown). This antibody also reacted with a protein in the apical membrane of the rat intestinal epithelium and in rat lung type II alveolar cells. Furthermore, this NH2-terminal antiserum identified similar-size protein bands (~80 and ~110 kDa) in mouse lung and intestine as COOH-terminal antibodies did in previous studies (2, 18, 26). Previous work identified ~110 and ~80 kDa band from 14-day-old rats (2), whereas in the current studies, we detected only ~80 kDa protein in adult rats. This protein size difference may be because of the differential posttranslational modification between suckling and adult rats. A similar observation has been reported in mice, where an ~88-kDa NaPi-IIb protein band was seen in suckling mice and a ~110-kDa NaPi-IIb was seen in adult mice. This difference was likely because of protein glycosylation (2).

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Fig. 7. Effect of β-estradiol on human NaPi-IIb gene promoter activity. Caco-2 cells were cotransfected with pGL3 basic (Basic) or human NaPi-IIb promoter constructs (pGL3−1,103, pGL3−563, pGL3−1,103, pGL3−2,783) plus pRL-CMV. β-Estradiol was applied 16 h before measuring promoter activities. The degree of induction is shown as the ratio of luciferase activity in vehicle-treated cells to β-estradiol-treated cells over luciferase activity in vehicle-treated cells. Results are means ± SE from 3 separate experiments. *P ≤ 0.01 for pGL3−2,783 vs. all others.
To decipher the molecular mechanism of estrogen regulation of intestinal NaPi-IIb gene expression, we used human intestinal epithelial (Caco-2) cells as an in vitro model. Our results demonstrate that endogenous NaPi-IIb gene expression was stimulated by estrogen treatment in Caco-2 cells and that this effect was inhibited by actinomycin D treatment. The increase in NaPi-IIb mRNA abundance in Caco-2 cells after estrogen treatment is similar to the increase observed in rats, suggesting that the increase in NaPi-IIb mRNA abundance induced by estrogen likely involves the synthesis of new NaPi-IIb mRNA in rats and in cells. Furthermore, transfection studies with human NaPi-IIb promoter constructs showed that estrogen increased NaPi-IIb gene promoter activity by ~36% in transiently transfected Caco-2 cells. When considered together, these data indicate that the effect of estrogen on intestinal NaPi-IIb gene expression can be mediated by increased transcriptional activation.

Transfection of cells with four NaPi-IIb gene promoter constructs (pGL3/−2,783 bp, pGL3/−1,103 bp, pGL3/−563 bp, and pGL3/−181 bp) resulted in significant reporter gene expression. However, only the longest promoter construct (pGL3/−2,783 bp) was responsive to estrogen treatment, suggesting that a putative estrogen response element(s) is located between 1,103 and 2,873 bp upstream of the transcriptional initiation site. Estrogen-responsive elements have been identified from many genes, including the pS2 (5), cbfa1 (25), keratin 19 (7), and calbindin-D-9K genes (11). By searching the human NaPi-IIb gene promoter region from −1103 bp to −2873 bp for putative estrogen-responsive elements, only one estrogen receptor half-binding site was identified, which is located at −2613 bp/−2609 bp region. This finding suggests that there may be a novel estrogen-responsive element present in this gene, or, alternatively, the estrogen response could be mediated by a trans-acting factor that acts independently of the estrogen receptor.

In summary, we show that estrogen treatment increases intestinal Na/Pi uptake in rats at least partially through increasing NaPi-IIb mRNA and protein abundance. We also demonstrate that estrogen increases NaPi-IIb mRNA expression in Caco-2 cells. Because actinomycin D treatment blocked estrogen-induced increases in NaPi-IIb mRNA expression in Caco-2 cells, we hypothesize that a transcriptional mechanism is likely involved.

**DISCLOSURES**

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