1,25-Dihydroxyvitamin D3-Independent Stimulatory Effect of Estrogen on the Expression of ECaC1 in the Kidney

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Abstract. Estrogen deficiency results in a negative Ca2+ balance and bone loss in postmenopausal women. In addition to bone, the intestine and kidney are potential sites for estrogen action and are involved in Ca2+ handling and regulation. The epithelial Ca2+ channel ECaC1 (or TRPV5) is the entry channel involved in active Ca2+ transport. Ca2+ entry is followed by cytosolic diffusion, facilitated by calbindin-D9k and/or calbindin-D28k, and active extrusion across the basolateral membrane by the Na+/Ca2+-exchanger (NCX1) and plasma membrane Ca2+-ATPase (PMCA1b). In this transcellular Ca2+ transport, ECaC1 probably represents the final regulatory target for hormonal control. The aim of this study was to determine whether 17β-estradiol (17β-E2) is involved in Ca2+ reabsorption via regulation of the expression of ECaC1. The ovariec- tomized rat model was used to investigate the regulation of ECaC1, at the mRNA and protein levels, by 17β-E2 replacement therapy. Using real-time quantitative PCR and immunohistochemical analyses, this study demonstrated that 17β-E2 treatment at pharmacologic doses increased renal mRNA levels of ECaC1, calbindin-D28k, NCX1, and PMCA1b and increased the protein abundance of ECaC1. Furthermore, the involvement of 1,25-dihydroxyvitamin D3 in the effects of 17β-E2 was examined in 25-hydroxyvitamin D3-1α-hydroxylase-knockout mice. Renal mRNA expression of calbindin-D28k, calbindin-D28k, NCX1, and PMCA1b was not significantly altered after 17β-E2 treatment. In contrast, ECaC1 mRNA and protein levels were both significantly upregulated. Moreover, 17β-E2 treatment partially restored serum Ca2+ levels, from 1.63 ± 0.06 to 2.03 ± 0.12 mM. In conclusion, this study suggests that 17β-E2 is positively involved in renal Ca2+ reabsorption via the upregulation of ECaC1, an effect independent of 1,25-dihydroxyvitamin D3.

Ca2+ homeostasis plays a key role in mammalian development and function and is maintained by the function of the small intestine, skeleton, and kidney. The classic hormones involved in Ca2+ homeostasis include 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], parathyroid hormone, and calcitonin (1,2). Estrogen is usually not considered a calciotropic hormone, but the idea that estrogen plays a role in Ca2+ homeostasis has been widely accepted (3). This involvement is clearly illustrated by the fact that estrogen deficiency results in a negative Ca2+ balance and bone loss among postmenopausal women (4). In addition to bone, two potential sites of estrogen action are the intestine and kidney, which are involved in Ca2+ handling and regulation.

There is increasing evidence that estrogen plays a physiologic role in the regulation of renal Ca2+ reabsorption. In vivo studies demonstrated that estrogen deficiency was associated with increased renal Ca2+-loss, which could be corrected with estrogen replacement therapy (5,6). Furthermore, there is evidence that estrogen receptors (ER) are also present in the kidney (7,8). ER localization studies have suggested both proximal and distal tubules as possible sites of action (9,10). However, the underlying mechanism by which estrogen might affect renal Ca2+ handling is still poorly understood. In addition, there is considerable disagreement regarding possible direct effects of estrogen on Ca2+ reabsorption versus indirect effects produced via actions on vitamin D metabolism.

The epithelial Ca2+ channel ECaC1 (or TRPV5), which is observed in renal distal convoluted tubule and connecting tubule cells, is the entry channel involved in transcellular Ca2+ transport (11). Ca2+ entry via ECaC1 is followed by cytosolic diffusion, facilitated by Ca2+-binding proteins (calbindin-D28k and/or calbindin-D9k), and active extrusion of Ca2+ across the basolateral membrane by a high-affinity plasma membrane Ca2+-ATPase (PMCA1b) and a Na+/Ca2+-exchanger (NCX1). In this active process, ECaC1 probably forms the final regulatory target for hormonal control (12,13). Therefore, ECaC1 could be a target for estrogen in the regulation of Ca2+ reabsorption.

The aim of this study was to determine whether estrogen acts on the distal part of the renal tubule by increasing the expression of ECaC1, which could result in increased Ca2+ reabsorp-
tion. We used the ovariecetomized (OVX) rat model of estrogen deficiency to investigate changes in ECaC1, at the mRNA and protein levels, with estrogen replacement therapy. The involvement of the important calcitropic hormone 1,25(OH)2D3 in estrogen effects was examined in 25-hydroxyvitamin D3/1α-hydroxylase (1α-OHase)-knockout mice, as a vitamin D-deficient model (14). This study indicates that estrogen upregulates ECaC1 expression in the kidney, thereby regulating Ca\(^{2+}\) reabsorption, and that estrogen exerts this effect independently of 1,25(OH)2D3.

Materials and Methods

Animals

**Experiment 1.** Twenty-five mature, virgin, female, Wistar rats (Hsd/Cpd:Wu, bred specific pathogen-free by Harlan, CPB, Zeist, The Netherlands), weighing 225 to 250 g, were housed individually in Macrolon cages, in a light- and temperature-controlled room (14-h light/10-h near-dark cycle, at 21 to 23°C). The rats received 16 g of standard pelleted food daily, and water was available ad libitum. On day 1 of the experiment, the rats were weighed and divided into five groups. After anesthesia induction, bilateral OVX or a sham operation was performed. Thereafter, rats received 17β-estradiol (17β-E2) (Sigma Chemical Co., St. Louis, MO) or vehicle (gelatin and mannitol), added to the pelleted food, each day. Sham-operated animals served as controls. OVX animals were given either the vehicle alone, 2 × 32 µg 17β-E2/d, 2 × 125 µg 17β-E2/d, or 2 × 500 µg 17β-E2/d. Treatment was started immediately after OVX and lasted for 7 d. Before the end of treatment, overnight urine samples were collected. On the last day, the rats were euthanized. Blood was collected from the abdominal aorta, the uterus was excised and weighed, and the kidneys were dissected and immediately frozen in liquid nitrogen.

**Experiment 2.** Homozygous, 9-wk-old, male, 1α-OHase-knockout mice (14) were housed individually in a light- and temperature-controlled room. Both deionized water and standard pelleted food were available ad libitum throughout the experiment. The mice were randomized into the following two groups (with each group consisting of four mice): homozygous mice and homozygous mice given infusions of 17β-E2; osmotic minipumps (model 1007D; Alzet, DURECT Corp., Cupertino, CA) were used for rate-controlled delivery of 17β-E2; these pumps release their contents at a rate of 0.5 µl/h for 7 d. The pumps were filled with the desired solution and implanted subcutaneously in the backs of the mice. The infusion dose for the 17β-E2-treated mice was 10 µg/d, and control mice received vehicle solution alone (15% ethanol/50% DMSO/35% water, vol/vol). After 7 d, mice were euthanized, blood was collected via orbital puncture, and kidneys were dissected and immediately frozen in liquid nitrogen. The animal ethics boards of the University of Nijmegen and Shriners Hospital for Children (Montreal, Quebec, Canada) approved all animal experimental procedures.

Analytical Procedures

Serum Ca\(^{2+}\) concentrations were analyzed by using a colorimetric assay kit, as described previously (15). Serum 17β-E2 levels were measured by using an extraction procedure with diethyl ether, followed by RIA (DPC, Los Angeles, CA) (16).

**RNA Isolation, Reverse Transcription, and Quantitative PCR**

Total RNA from kidney cortex was isolated by using TriZol reagent (Life Technologies, Breda, The Netherlands), according to the protocol described by the manufacturer. RNA was treated with DNase, to prevent genomic DNA contamination, and was finally resuspended in diethylpyrocarbonate-treated water filtered with a MilliQ system (Millipore, Bedford, MA). Total RNA (2 µg) was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Life Technologies), as described previously (17). Expression levels of renal ECaC1, calbindin-D28K, calbindin-D9K, NCX1, and PMCA1b mRNA were quantified by real-time quantitative PCR, using an ABI Prism 7700 sequence detection system (PE Biosystems, Rotkreuz, Switzerland). The expression level of hypoxanthine-guanine phosphoribosyl transferase was used as an internal control, to normalize differences in RNA extractions, the degree of RNA degradation, and reverse transcription efficiencies. Primers and probes targeting the genes of interest were designed by using Primer Express software (Applied Biosystems, Foster City, CA) and are listed in Table 1. The 3'-ends of the probes were labeled with the quencher dye 6-carboxyfluorescein (Biolegio, Malden, The Netherlands).

Immunohistochemical Analyses

Seven-micron sections of frozen kidney tissue were cut with a cryotome and collected on SuperFrost/Plus-coated (Menzel-Glazer, Germany) glass slides. For ECaC1 staining, sections were incubated in boiled citrate buffer (0.01 M sodium citrate, 0.01 M citric acid, pH 6), cooled for 30 min, and washed three times with TN buffer (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5). For immunoperoxidase staining, sections were incubated for 30 min at room temperature in TN buffer containing 0.3% (vol/vol) H2 O2 and were washed three times with TN buffer. Sections were then incubated for 30 min in TMB buffer (TN buffer containing 0.5%, wt/vol, blocking reagent; NEN Life Science Products, Perkin Elmer, Boston, MA). Subsequently, sections were incubated for 16 h at 4°C in TMB buffer containing affinity-purified guinea pig anti-ECaC1 antiserum (1:200) (11). Sections were washed three times with TNT buffer (TN buffer containing 0.05%, vol/vol, Tween 20) and then incubated for 1 h at room temperature with biotin-labeled, affinity-purified, goat anti-guinea pig IgG (1:2000; Sigma Chemical Co., St. Louis, MO). After washing with TNT buffer, sections were incubated with streptavidin-horseradish peroxidase (1:100; NEN Life Science Products) for 30 min at room temperature. Sections were washed three times with TNT buffer and incubated for 7 min with fluorescein tyramide in amplification diluent (1:50; NEN Life Science Products). Sections were then washed, dehydrated in 50 to 100% (vol/vol) methanol, and mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 2.5% (vol/vol) NaNO3. Photographs were taken with a Bio-Rad MRC 1000 confocal laser scanning microscope. The anti-ECaC1 antibody used in this study has been extensively characterized but is, unfortunately, not able to be used for immunoblotting (17). For semiquantitative assessment of ECaC1 protein expression, the relative amounts of immunopositive tubules in 10 to 15 randomly selected microscopic fields were determined for each animal, using a Zeiss Axioskop microscope (Zeiss, Thornwood, NY) equipped for epifluorescence illumination.

Statistical Analyses

Values are expressed as mean ± SEM. Differences between groups were tested by using one-way ANOVA and were further evaluated by using Fisher’s multiple-comparison procedure (18). Differences in means with P < 0.05 were considered statistically significant. All analyses were performed by using the Statview statistical package.
Sequences of primers and Taqman probes for real-time quantitative PCR

Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tr>
<td>HPRT R</td>
<td>5'-TATCAGACTGAAGAGCTACTGTAATGACC-3'</td>
<td>/H11032</td>
<td>H11032</td>
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<td>/H11032</td>
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<td>/H11032</td>
</tr>
<tr>
<td>M5</td>
<td>5'-TTACCAGTGTCAATTATATCTTCAACAATC-3'</td>
<td>/H11032</td>
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<tr>
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<td></td>
<td>/H11032</td>
<td>/H11032</td>
<td>/H11032</td>
</tr>
<tr>
<td>CaBP-D28k R</td>
<td>5'-GGAAAGCTGGAGCTGACAGAGAT-3'</td>
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<td>/H11032</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>/H11032</td>
<td>/H11032</td>
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</tr>
<tr>
<td>PMCA1b R</td>
<td>5'-CAGCTGAAAGGCTTCCCGCCAAA-3'</td>
<td>/H11032</td>
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**Results**

**Effects of OVX and 17β-E₂ Treatment on Rats**

Age-matched, untreated, OVX rats weighed significantly more than rats in the sham-operated group (Table 2). In contrast, 17β-E₂ treatment induced significant reductions in weight, with the greatest decrease being observed for the OVX/high-dose 17β-E₂-treated rats (Table 2). OVX resulted in the expected atrophy of the uterus, which was prevented by 17β-E₂ therapy in OVX/high-dose 17β-E₂-treated rats (Table 2). Serum 17β-E₂ levels were reduced in untreated OVX rats, confirming OVX, whereas supplementation with the highest 17β-E₂ dose resulted in significantly higher serum 17β-E₂ levels (Table 2). 17β-E₂ treatment reduced serum Ca²⁺ levels, resulting in slightly but significantly lower serum Ca²⁺ levels in the OVX/high-dose 17β-E₂-treated group.

**Effect of 17β-E₂ on ECaC1 Expression in Rat Kidneys**

To investigate the effect of 17β-E₂ on ECaC1 mRNA expression, real-time quantitative PCR was performed with total RNA isolated from kidney cortex (Figure 1A). The data demonstrated that 17β-E₂ supplementation, using the highest dosage, for estrogen-depleted rats resulted in a 2.5-fold increase in ECaC1 mRNA expression, compared with OVX rats. The expression levels of other Ca²⁺-transport proteins involved in transcellular Ca²⁺ transport, namely calbindin-D28k, NCX1, and PMCA1b, were also determined. More than twofold up-regulation of calbindin-D28k mRNA expression was observed in OVX/high-dose 17β-E₂-treated rats, compared with OVX rats (Figure 1B). In addition, significant increases in the expression of both NCX1 (5.5-fold) and PMCA1b (1.5-fold) mRNA in OVX/medium-dose 17β-E₂-treateed rats, compared with OVX rats, were demonstrated (Figure 1, C and D). Next, the abundance of ECaC1 protein in kidneys was examined. Figure 2A presents representative immunofluorescence labeling of distal tubules in sections of kidney cortex from OVX and OVX/high-dose 17β-E₂-treated rats. More ECaC1 protein was detected in tissue from the supplemented rats, as indicated by the increased staining in the kidney cortex. In these immunopositive tubules, ECaC1 was localized to the apical membrane of distal tubular segments. For semiquantitative assessment of ECaC1 protein expression, the relative amounts of immunopositive tubules in 10 to 15 randomly selected microscopic fields were counted for each kidney cortex section. Figure 2B presents the average values for each experimental group. The levels of ECaC1 expression in OVX/medium-dose 17β-E₂-treated and OVX/high-dose 17β-E₂-treated rats were significantly higher than those in OVX rats.

**17β-E₂-Induced Increases in Plasma Ca²⁺ Levels in 1,25(OH)₂D₃-Deficient Mice**

One of the characteristics of the homozygous 1α-OHase-knockout mouse is growth retardation, as illustrated by their low
Effects of ovariectomy (OVX) and 17β-E2 supplementation on the mRNA expression levels of ECaC1 and other Ca2+-transport proteins in rat kidneys. mRNA expression levels were measured by using real-time quantitative PCR, as described in Materials and Methods. The expression levels of ECaC1 (A), calbindin-D28K (B), plasma membrane Ca2+-ATPase (PMCA1b) (C), and Na+/Ca2+-exchanger (NCX1) (D) for the different experimental groups are presented relative to levels in sham-treated rats. +E2L, supplemented with 2 × 32 μg 17β-E2/d; +E2M, supplemented with 2 × 125 μg 17β-E2/d; +E2H, supplemented with 2 × 500 μg 17β-E2/d. Data are presented as means ± SEM (n = 5). *P < 0.01 versus all groups; †P < 0.05 versus OVX and OVX plus low-dose 17β-E2; ‡P < 0.05 versus all groups.

Figure 2. Effects of OVX and 17β-E2 supplementation on the protein expression levels of ECaC1 in rat kidneys. (A) Immunoperoxidase staining for ECaC1 in kidney cortex sections from OVX rats (top) and OVX/high-dose 17β-E2-treated rats (bottom). (B) Protein expression levels, calculated by counting the relative amounts of positive tubules in 10 to 15 views in each immunostained section. +E2L, supplemented with 2 × 32 μg 17β-E2/d; +E2M, supplemented with 2 × 125 μg 17β-E2/d; +E2H, supplemented with 2 × 500 μg 17β-E2/d. Data are presented as means ± SEM (n = 4). *P < 0.001 versus all groups; †P < 0.01 versus OVX and OVX plus low-dose 17β-E2.

Table 2. Effects of OVX and 17β-E2 supplementation in rats

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 5)</th>
<th>OVX (n = 5)</th>
<th>OVX + E2L (n = 5)</th>
<th>OVX + E2M (n = 5)</th>
<th>OVX + E2H (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g) beginning</td>
<td>207 ± 4</td>
<td>205 ± 4</td>
<td>207 ± 3</td>
<td>209 ± 4</td>
<td>204 ± 2</td>
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<tr>
<td>final</td>
<td>203 ± 4</td>
<td>215 ± 3b</td>
<td>207 ± 4</td>
<td>202 ± 4c</td>
<td>189 ± 4d</td>
</tr>
<tr>
<td>Uterus weight (g)</td>
<td>0.51 ± 0.07</td>
<td>0.23 ± 0.04b</td>
<td>0.21 ± 0.01</td>
<td>0.39 ± 0.04</td>
<td>0.66 ± 0.13g</td>
</tr>
<tr>
<td>Serum level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17β-E2 (pg/ml)</td>
<td>11 ± 6</td>
<td>5 ± 3</td>
<td>5 ± 1</td>
<td>12 ± 2</td>
<td>64 ± 6f</td>
</tr>
<tr>
<td>Ca2+ (mM)</td>
<td>2.38 ± 0.02</td>
<td>2.40 ± 0.03</td>
<td>2.39 ± 0.02</td>
<td>2.36 ± 0.04</td>
<td>2.31 ± 0.02c</td>
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</table>

Note: OVX, ovariectomized; 17β-E2, 17β-estradiol; +E2L, supplemented with 2 × 32 μg 17β-E2/d; +E2M, supplemented with 2 × 125 μg 17β-E2/d; +E2H, supplemented with 2 × 500 μg 17β-E2/d. Data are presented as mean ± SEM.

b P < 0.05 versus sham.

c P < 0.05 versus OVX.

d P < 0.05 versus all groups.

OVX and OVX plus medium-dose 17β-E2.

1P < 0.0001 versus all groups.
Table 3. Effects of 17β-E2 in 1α-OHase-knockout mice

<table>
<thead>
<tr>
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<th>Control (n = 4)</th>
<th>17β-E2 (n = 4)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
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<td></td>
</tr>
<tr>
<td>beginning</td>
<td>15 ± 2</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>final</td>
<td>15 ± 2</td>
<td>14 ± 1</td>
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<tr>
<td>Serum level</td>
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<tr>
<td>17β-E2 (pg/ml)</td>
<td>ND</td>
<td>67 ± 12</td>
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<tr>
<td>Ca²⁺ (mM), before</td>
<td>1.54 ± 0.06</td>
<td>1.63 ± 0.06</td>
</tr>
<tr>
<td>Ca²⁺ (mM), after</td>
<td>1.69 ± 0.10</td>
<td>2.03 ± 0.12</td>
</tr>
</tbody>
</table>

*1α-OHase, 25-hydroxyvitamin D₃-1α-hydroxylase; control, 1α-OHase-knockout mice; 17β-E2, 1α-OHase-knockout mice supplemented with 10 μg 17β-E₂/d; ND, not detectable. Data are presented as means ± SEM.

1,25(OH)₂D₃-Independent Effect of 17β-E₂ on ECaC1 Expression

To investigate the involvement of 1,25(OH)₂D₃ in the stimulatory effect of 17β-E₂ supplementation on the expression of ECaC1 and other renal Ca²⁺-transport proteins, control and 17β-E₂-treated 1α-OHase-knockout mice were used. A 2.5-fold increase in ECaC1 transcript levels in the kidney cortex of 17β-E₂-treated mice, compared with control mice, was observed (Figure 3A). However, in contrast to the upregulation of ECaC1, no significant changes in the expression of calbindin-D₂₈K, NCX1, or PMCA1b were observed after treatment (Figure 3, B to E). Immunohistochemical analyses confirmed the increase in ECaC1 expression, after 17β-E₂ treatment, at the protein level, as indicated by the increased staining in the kidney cortex of 17β-E₂-treated mice, compared with control mice (Figure 4A). Semiquantitative assessment of the relative amounts of immunopositive tubules demonstrated a significant increase in the expression of ECaC1 protein after 17β-E₂ treatment (Figure 4B).

Discussion

The main conclusion from this study is that estrogen upregulates the expression of ECaC1 in the kidney, in a 1,25(OH)₂D₃-independent manner. By upregulating ECaC1 expression, as the rate-limiting step in transcellular transport, estrogen could be positively involved in Ca²⁺ reabsorption. Estrogen deficiencies after menopause result in bone loss, which is associated with increases in plasma and urinary Ca²⁺ levels (4). It has been generally reported that the increases in plasma and urinary Ca²⁺ levels are secondary to increases in bone resorption. However, some studies have demonstrated that the increases in urinary Ca²⁺ levels after menopause are not attributable to increases in the filtered load, suggesting that estrogen also has an effect on renal Ca²⁺ handling (5,6). This study identified ECaC1 as a molecular target for the action of estrogen in the kidney.

Estrogen replacement therapy for the OVX rats, at pharmacologic doses, resulted in significant upregulation of ECaC1 mRNA and protein expression. ECaC1 protein was observed exclusively at the apical side of distal tubular cells, in agreement with previous studies (17,20). The increased mRNA levels after 17β-E₂ treatment could be attributable to enhanced transcriptional activity or mRNA stabilization. In addition to increases in mRNA abundance, translational regulation of ECaC1 might occur, ultimately resulting in increased channel activity at the apical cell surface. In general, estrogen activates the ER, which subsequently regulates gene transcription by binding to the classic estrogen response elements. ER have been observed in the kidney, especially in the proximal tubules, where 17β-E₂ is retained in the nuclei (10). However, Davidoff et al. (9) determined, using autoradiography, that ER are also localized (albeit to a lesser extent) in the distal tubules, the site of ECaC1 expression. Interestingly, Weber et al. (21) recently described an estrogen response element in the promoter se-
There is evidence that an estrogen-responsive promoter exists
cally upregulated, suggesting that this requirement is fulfilled.
tosolic Ca\(_{2+}\) via activator protein 1 (AP-1) binding sites or GC-rich stimu-
tional activation by the estrogen-liganded ER can be mediated
not present in the mouse ECaC1 gene. Alternatively, transcrip-
tory effect of 17\(\beta\)-E\(_2\) on transcellular Ca\(_{2+}\) reabsorption, independent of
(27). Criddle et al. (28) demonstrated that estrogen treatment
increased renal calbindin-D\(_{28k}\) mRNA levels in OVX rats,
several hours after administration. In addition, tremendous
upregulation of NCX1 and a slight but significant increase in
the levels of PMCA1b (both of which are members of the
basolateral extrusion system) were observed. In agreement
with this finding, it has been suggested that Ca\(_{2+}\) extrusion
across the basolateral membrane is mediated primarily by
NCX1 (13,29). Taken together, these findings suggest a posi-
tive effect of 17\(\beta\)-E\(_2\) on renal tubular reabsorption of Ca\(_{2+}\).
However, 17\(\beta\)-E\(_2\) treatment resulted in decreased serum Ca\(_{2+}\)
levels. This effect of estrogen therapy on serum Ca\(_{2+}\) levels
was previously observed among human subjects (30). This
apparent discrepancy can be explained on the basis of the
increased Ca\(_{2+}\) requirements for estrogen-deficient animals.
Correction of estrogen deficiencies results in decreased bone
resorption and increased formation, causing slight decreases in
plasma Ca\(_{2+}\) concentrations (3).

The relationship between estrogen actions in the kidney and
vitamin D metabolism is still unclear. 1,25(OH\(_{2}\))D\(_3\) is a major
regulator of Ca\(_{2+}\) homeostasis, and the kidney is the principal site
of its synthesis (31). In the literature, conflicting data have been
presented regarding direct versus indirect effects of 17\(\beta\)-E\(_2\) on
Ca\(_{2+}\) reabsorption. Some studies demonstrated that estrogen treat-
ment increased 1,25(OH\(_{2}\))D\(_3\) levels among postmenopausal
women (32). One study demonstrated that 17\(\beta\)-E\(_2\) was retained in
the cell nuclei of proximal tubules, where the synthesis of
1,25(OH\(_{2}\))D\(_3\) takes place (10). With the use of cultured opossum
kidney cells, a suppressive effect of 17\(\beta\)-E\(_2\) on \(\alpha\)-OHase activity
was demonstrated (33). Among postmenopausal women, how-
ever, estrogen replacement therapy resulted in unchanged or in-
creased renal \(\alpha\)-OHase activity (30,34). In previous studies, it
was demonstrated that Ca\(_{2+}\) reabsorption was stimulated by
1,25(OH\(_{2}\))D\(_3\) (35,36). Furthermore, 1,25(OH\(_{2}\))D\(_3\) enhances the
renal expression of ECaC1 and Ca\(_{2+}\)-transport proteins (calbindin-
D\(_{28k}\), NCX1, and PMCA1b), as demonstrated in previous
studies (20,29,36,37). In contrast, some studies suggested a
direct effect of 17\(\beta\)-E\(_2\) on Ca\(_{2+}\) reabsorption, independent of
vitamin D (3,28).

\(\alpha\)-OHase-knockout mice represent an ideal animal model
for studies of the effects of 17\(\beta\)-E\(_2\) on transcellular Ca\(_{2+}\)
transport independent of 1,25(OH\(_{2}\))D\(_3\) as demonstrated in this
study and reported by the two laboratories that engineered the
\(\alpha\)-OHase-knockout mice, these animals exhibit severe hy-
pocalcemia (14,19). Treatment with 17\(\beta\)-E\(_2\), at pharmacologic
doses, results in significant increases in serum Ca\(_{2+}\) levels (to
subnormal concentrations) and increases in the expression of
ECaC1. These findings suggest a positive effect of 17\(\beta\)-E\(_2\) on
plasma Ca\(_{2+}\) levels, which might be attributable to increased
renal Ca\(_{2+}\) reabsorption through ECaC1, independently of
1,25(OH\(_{2}\))D\(_3\). Therefore, 17\(\beta\)-E\(_2\) could be directly involved in
Ca\(_{2+}\) reabsorption via upregulation of ECaC1 gene expression
in the absence of circulating 1,25(OH\(_{2}\))D\(_3\). In contrast, the
expression of the other transport proteins remained unchanged,
indicating 1,25(OH\(_{2}\))D\(_3\) dependence for the regulation of cal-
bindins, NCX1, and PMCA1b. An explanation for this discrep-
ancy could be that, in addition to 17\(\beta\)-E\(_2\), 1,25(OH\(_{2}\))D\(_3\) is

![Image](https://via.placeholder.com/150)
required for optimal stimulation of Ca\(^{2+}\) transport, eventually leading to normalized Ca\(^{2+}\) levels. Another explanation could be that, in addition to upregulated expression of ECaC1, sufficient calbindins and extrusion proteins are present to facilitate active transport, leading to normalization of serum Ca\(^{2+}\) levels. This is in accord with previous studies that suggested that ECaC1 represents the rate-limiting step in active Ca\(^{2+}\) transport (12,13). Also, the intestine must be considered to contribute to the increase in serum Ca\(^{2+}\) levels. It was previously demonstrated that estrogen has a positive effect on intestinal Ca\(^{2+}\) absorption, and it has been suggested that this effect is independent of 1,25(OH)\(_2\)D\(_3\) (16,38,39). ECaC1 and its homolog ECaC2 are present in the duodenum, as are other transport proteins necessary for active Ca\(^{2+}\) transport (11,17).

In addition to direct effects on bone, estrogen deficiencies might directly affect renal and intestinal Ca\(^{2+}\) handling, resulting in a continuing negative Ca\(^{2+}\) balance. Furthermore, interactions with other calcitropic hormones in these tissues must be considered. Because of the high prevalence of osteoporosis, intensive investigations have been performed to clarify the underlying pathophysiologic mechanisms. This study provides evidence that 17β-E\(_2\) is positively involved in renal Ca\(^{2+}\) reabsorption via the upregulation of ECaC1, an effect independent of 1,25(OH)\(_2\)D\(_3\). Future detailed studies of the cellular mechanisms of estrogen actions in bone, kidney, and intestine should lead to novel insights regarding the treatment of estrogen-associated disorders.

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