

# 1,25-Dihydroxyvitamin D<sub>3</sub>-Independent Stimulatory Effect of Estrogen on the Expression of ECaC1 in the Kidney

MONIQUE VAN ABEL,\* JOOST G. J. HOENDEROP,\* OLIVIER DARDENNE,<sup>†</sup> RENÉ ST. ARNAUD,<sup>‡</sup> CAREL H. VAN OS,\* HANS J. P. T. M. VAN LEEUWEN,<sup>‡</sup> and RENÉ J. M. BINDELS\*

\*Department of Cell Physiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, Nijmegen, The Netherlands; <sup>†</sup>Genetics Unit, Shriners Hospital for Children, Montreal, Quebec, Canada; and <sup>‡</sup>Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.

**Abstract.** Estrogen deficiency results in a negative Ca<sup>2+</sup> 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 Ca<sup>2+</sup> handling and regulation. The epithelial Ca<sup>2+</sup> channel ECaC1 (or TRPV5) is the entry channel involved in active Ca<sup>2+</sup> transport. Ca<sup>2+</sup> entry is followed by cytosolic diffusion, facilitated by calbindin-D<sub>28K</sub> and/or calbindin-D<sub>9K</sub>, and active extrusion across the basolateral membrane by the Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger (NCX1) and plasma membrane Ca<sup>2+</sup>-ATPase (PMCA1b). In this transcellular Ca<sup>2+</sup> transport, ECaC1 probably represents the final regulatory target for hormonal control. The aim of this study was to determine whether 17β-estradiol (17β-E<sub>2</sub>) is involved in Ca<sup>2+</sup> reabsorption via regulation of the expression of ECaC1. The ovariectomized rat model was used to investigate the regulation of ECaC1, at the mRNA and protein levels, by 17β-E<sub>2</sub>

replacement therapy. Using real-time quantitative PCR and immunohistochemical analyses, this study demonstrated that 17β-E<sub>2</sub> treatment at pharmacologic doses increased renal mRNA levels of ECaC1, calbindin-D<sub>28K</sub>, NCX1, and PMCA1b and increased the protein abundance of ECaC1. Furthermore, the involvement of 1,25-dihydroxyvitamin D<sub>3</sub> in the effects of 17β-E<sub>2</sub> was examined in 25-hydroxyvitamin D<sub>3</sub>-1α-hydroxylase-knockout mice. Renal mRNA expression of calbindin-D<sub>9K</sub>, calbindin-D<sub>28K</sub>, NCX1, and PMCA1b was not significantly altered after 17β-E<sub>2</sub> treatment. In contrast, ECaC1 mRNA and protein levels were both significantly upregulated. Moreover, 17β-E<sub>2</sub> treatment partially restored serum Ca<sup>2+</sup> levels, from 1.63 ± 0.06 to 2.03 ± 0.12 mM. In conclusion, this study suggests that 17β-E<sub>2</sub> is positively involved in renal Ca<sup>2+</sup> reabsorption via the upregulation of ECaC1, an effect independent of 1,25-dihydroxyvitamin D<sub>3</sub>.

Ca<sup>2+</sup> 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 Ca<sup>2+</sup> homeostasis include 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], parathyroid hormone, and calcitonin (1,2). Estrogen is usually not considered a calciotropic hormone, but the idea that estrogen plays a role in Ca<sup>2+</sup> homeostasis has been widely accepted (3). This involvement is clearly illustrated by the fact that estrogen deficiency results in a negative Ca<sup>2+</sup> 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 Ca<sup>2+</sup> handling and regulation.

There is increasing evidence that estrogen plays a physiologic role in the regulation of renal Ca<sup>2+</sup> reabsorption. *In vivo*

studies demonstrated that estrogen deficiency was associated with increased renal Ca<sup>2+</sup> 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 Ca<sup>2+</sup> handling is still poorly understood. In addition, there is considerable disagreement regarding possible direct effects of estrogen on Ca<sup>2+</sup> reabsorption *versus* indirect effects produced via actions on vitamin D metabolism.

The epithelial Ca<sup>2+</sup> channel ECaC1 (or TRPV5), which is observed in renal distal convoluted tubule and connecting tubule cells, is the entry channel involved in transcellular Ca<sup>2+</sup> transport (11). Ca<sup>2+</sup> entry via ECaC1 is followed by cytosolic diffusion, facilitated by Ca<sup>2+</sup>-binding proteins (calbindin-D<sub>28K</sub> and/or calbindin-D<sub>9K</sub>), and active extrusion of Ca<sup>2+</sup> across the basolateral membrane by a high-affinity plasma membrane Ca<sup>2+</sup>-ATPase (PMCA1b) and a Na<sup>+</sup>/Ca<sup>2+</sup>-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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> reabsorp-

Received April 3, 2002. Accepted April 29, 2002.

Correspondence to Dr. René J. M. Bindels, 160 Cell Physiology, University Medical Center Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands. Phone: +31-24-3614211; Fax: +31-24-3616413; E-mail: r.bindels@ncmls.kun.nl

1046-6673/1308-2102

Journal of the American Society of Nephrology  
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DOI: 10.1097/01.ASN.0000022423.34922.2A

tion. We used the ovariectomized (OVX) rat model of estrogen deficiency to investigate changes in ECaCl<sub>1</sub>, at the mRNA and protein levels, with estrogen replacement therapy. The involvement of the important calcitropic hormone 1,25(OH)<sub>2</sub>D<sub>3</sub> in estrogen effects was examined in 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase)-knockout mice, as a vitamin D-deficient model (14). This study indicates that estrogen upregulates ECaCl<sub>1</sub> expression in the kidney, thereby regulating Ca<sup>2+</sup> reabsorption, and that estrogen exerts this effect independently of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

## 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 17 $\beta$ -estradiol (17 $\beta$ -E<sub>2</sub>) (Sigma Chemical Co., St. Louis, MO) or vehicle (gelatin and mannitol), added to the pelleted food, each day. Sham-operated animals served as control animals. OVX animals were given either the vehicle alone, 2  $\times$  32  $\mu$ g 17 $\beta$ -E<sub>2</sub>/d, 2  $\times$  125  $\mu$ g 17 $\beta$ -E<sub>2</sub>/d, or 2  $\times$  500  $\mu$ g 17 $\beta$ -E<sub>2</sub>/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, 1 $\alpha$ -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 17 $\beta$ -E<sub>2</sub>. Osmotic minipumps (model 1007D; Alzet, DURECT Corp., Cupertino, CA) were used for rate-controlled delivery of 17 $\beta$ -E<sub>2</sub>; these pumps release their contents at a rate of 0.5  $\mu$ l/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 17 $\beta$ -E<sub>2</sub>-treated mice was 10  $\mu$ g/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<sup>2+</sup> concentrations were analyzed by using a colorimetric assay kit, as described previously (15). Serum 17 $\beta$ -E<sub>2</sub> 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  $\mu$ g) was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Life Technologies), as described previously (17). Expression levels of renal ECaCl<sub>1</sub>, calbindin-D<sub>28K</sub>, calbindin-D<sub>9K</sub>, 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-carboxytetramethylrhodamine. The 5'-ends of all probes were labeled with the reporter 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 ECaCl<sub>1</sub> 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) H<sub>2</sub>O<sub>2</sub> and were washed three times with TN buffer. Sections were then incubated for 30 min in TNB 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 TNB buffer containing affinity-purified guinea pig anti-ECaCl<sub>1</sub> 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% (wt/vol) NaN<sub>3</sub>. Photographs were taken with a Bio-Rad MRC 1000 confocal laser scanning microscope. The anti-ECaCl<sub>1</sub> antibody used in this study has been extensively characterized but is, unfortunately, not able to be used for immunoblotting (17). For semiquantitative assessment of ECaCl<sub>1</sub> 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  $\pm$  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

Table 1. Sequences of primers and Taqman probes for real-time quantitative PCR<sup>a</sup>

Gene	Forward Primer	Reverse Primer	Probe
HPRT	R 5'-TATCAGACTGAAGAGCTACTGTAATGACC-3'	5'-TTACCAGTGTCAATTATATCTTCAACAATC-3'	5'-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3'
	M 5'-TTATCAGACTGAAGAGCTACTGTAATGATC-3'	5'-TTACCAGTGTCAATTATATCTTCAACAATC-3'	5'-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3'
ECaC1	R 5'-CTTAGCGGGTTGAACACCA-3'	5'-TTGCAGAACACAGAGCCCTCA-3'	5'-TGTCTTCAGATAGTTGTTCTTGTACTTCCCTCTGT-3'
	M 5'-CGTTGGTCTTACGGGTTGAAC-3'	5'-GTTTGGAGAACACAGAGCCCTCA-3'	5'-TGTCTTCAGATAGTTGTTCTTGTACTTCCCTCTGT-3'
CaBP-D28k	R 5'-GGAAGCTGGAGCTGACAGAGAT-3'	5'-TGAACCTTTTCCACACATTTGAT-3'	5'-ACCAGTGCAGGAAAATTCCTTCTTAAATCCA-3'
	M 5'-AACTGACAGAGATGGCCAGGTTA-3'	5'-TGAACCTTTTCCACACATTTGAT-3'	5'-ACCAGTGCAGGAAAATTCCTTCTTAAATCCA-3'
CaBP-D9k	M 5'-CTTCAGAAATGAAGCATTTT-3'	5'-CTCCATCGCCATCTTATCCA-3'	5'-CAAAAATATGAGCCAGGAAGGCGA-3'
PMCA1b	R 5'-CGCCATCTCTGCACAAT-3'	5'-CAGCCATTGCTCTATGAAAAGTTC-3'	5'-CAGCTGAAAAGGCTCCCGCCAAA-3'
	M 5'-CGCCATCTCTGCACAAT-3'	5'-CAGCCATTGCTCTATGAAAAGTTC-3'	5'-CAGCTGAAAAGGCTCCCGCCAAA-3'
NCX1	R 5'-TCCCTATAAAACCAATGAAGGCACA-3'	5'-TTTCTCATACTCTCGTCATCGATT-3'	5'-ACCTTGACTGATATGTTTGTGACTATTTTCATCATCTGGA-3'
	M 5'-TCCCTACAAAACCAATGAAGGCACA-3'	5'-TTTCTCATACTCTCGTCATCGATT-3'	5'-ACCTTGACTGATATGTTTGTGACTATTTTCATCATCTGGA-3'

<sup>a</sup> PCR primers and fluorescent probes (5'-6-carboxyfluorescein- and 3'-6-carboxyfluorescein-labeled) were designed by using the computer program Primer Express (Applied Biosystems) and were purchased from Biologig. HPRT, hypoxanthine-guanine phosphoribosyl transferase; ECaC1, epithelial Ca<sup>2+</sup> channel 1; CaBP, calbindin; PMCA1b, plasma membrane Ca<sup>2+</sup>-ATPase; NCX1, Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger; R, rat; M, mouse.

(Power PC version 4.51; Statview, Berkeley, CA) on a Macintosh computer.

## Results

### Effects of OVX and 17β-E<sub>2</sub> Treatment on Rats

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

### Effect of 17β-E<sub>2</sub> on ECaC1 Expression in Rat Kidneys

To investigate the effect of 17β-E<sub>2</sub> on ECaC1 mRNA expression, real-time quantitative PCR was performed with total RNA isolated from kidney cortex (Figure 1A). The data demonstrated that 17β-E<sub>2</sub> 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 Ca<sup>2+</sup>-transport proteins involved in transcellular Ca<sup>2+</sup> transport, namely calbindin-D<sub>28K</sub>, NCX1, and PMCA1b, were also determined. More than twofold up-regulation of calbindin-D<sub>28K</sub> mRNA expression was observed in OVX/high-dose 17β-E<sub>2</sub>-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-E<sub>2</sub>-treated 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-E<sub>2</sub>-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-E<sub>2</sub>-treated and OVX/high-dose 17β-E<sub>2</sub>-treated rats were significantly higher than those in OVX rats.

### 17β-E<sub>2</sub>-Induced Increases in Plasma Ca<sup>2+</sup> Levels in 1,25(OH)<sub>2</sub>D<sub>3</sub>-Deficient Mice

One of the characteristics of the homozygous 1α-OHase-knockout mice is growth retardation, as illustrated by their low



Table 2. Effects of OVX and 17β-E<sub>2</sub> supplementation in rats<sup>a</sup>

	Sham (n = 5)	OVX (n = 5)	OVX + E <sub>2</sub> L (n = 5)	OVX + E <sub>2</sub> M (n = 5)	OVX + E <sub>2</sub> H (n = 5)
Body weight (g)					
beginning	207 ± 4	205 ± 4	207 ± 3	209 ± 4	204 ± 2
final	203 ± 4	215 ± 3 <sup>b</sup>	207 ± 4	202 ± 4 <sup>c</sup>	189 ± 4 <sup>d</sup>
Uterus weight (g)	0.51 ± 0.07	0.23 ± 0.04 <sup>b</sup>	0.21 ± 0.01	0.39 ± 0.04	0.66 ± 0.13 <sup>e</sup>
Serum level					
17β-E <sub>2</sub> (pg/ml)	11 ± 6	5 ± 3	5 ± 1	12 ± 2	64 ± 6 <sup>f</sup>
Ca <sup>2+</sup> (mM)	2.38 ± 0.02	2.40 ± 0.03	2.39 ± 0.02	2.36 ± 0.04	2.31 ± 0.02 <sup>e</sup>

<sup>a</sup> OVX, ovariectomized; 17β-E<sub>2</sub>, 17β-estradiol; +E<sub>2</sub>L, supplemented with 2 × 32 μg 17β-E<sub>2</sub>/d; +E<sub>2</sub>M, supplemented with 2 × 125 μg 17β-E<sub>2</sub>/d; +E<sub>2</sub>H, supplemented with 2 × 500 μg 17β-E<sub>2</sub>/d. Data are presented as mean ± SEM.

<sup>b</sup> P < 0.05 versus sham.

<sup>c</sup> P < 0.05 versus OVX.

<sup>d</sup> P < 0.05 versus all groups.

<sup>e</sup> P < 0.05 versus OVX, OVX plus low-dose 17β-E<sub>2</sub>, and OVX plus medium-dose 17β-E<sub>2</sub>.

<sup>f</sup> P < 0.0001 versus all groups.

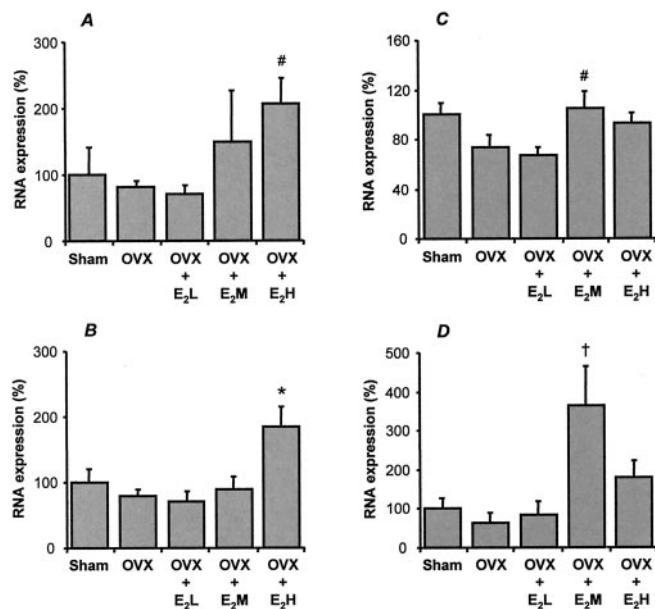


Figure 1. Effects of ovariectomy (OVX) and 17β-estradiol (17β-E<sub>2</sub>) supplementation on the mRNA expression levels of ECaC1 and other Ca<sup>2+</sup>-transport proteins in rat kidneys. mRNA expression levels were measured by using real-time quantitative PCR, as described in Materials and Methods. The expression levels of ECaC1 (A), calbindin-D<sub>28K</sub> (B), plasma membrane Ca<sup>2+</sup>-ATPase (PMCA1b) (C), and Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger (NCX1) (D) for the different experimental groups are presented relative to levels in sham-treated rats. +E<sub>2</sub>L, supplemented with 2 × 32 μg 17β-E<sub>2</sub>/d; +E<sub>2</sub>M, supplemented with 2 × 125 μg 17β-E<sub>2</sub>/d; +E<sub>2</sub>H, supplemented with 2 × 500 μg 17β-E<sub>2</sub>/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-E<sub>2</sub>; †P < 0.05 versus all groups.

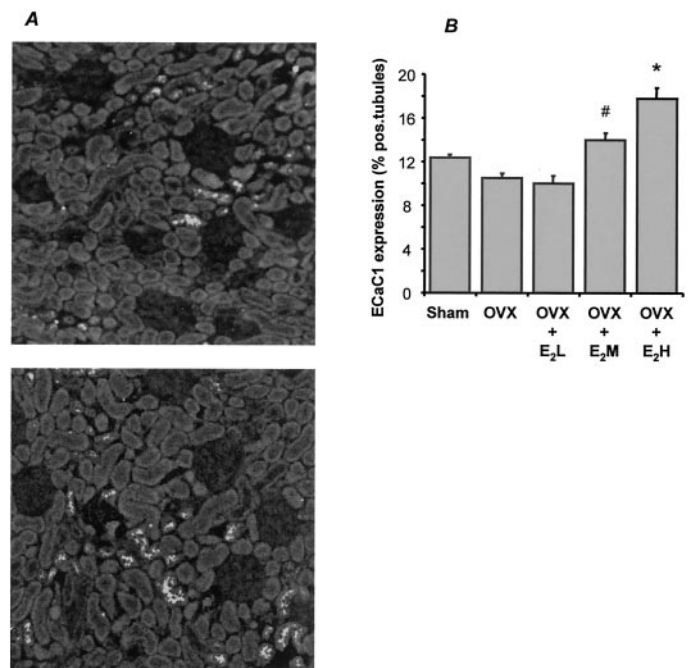


Figure 2. Effects of OVX and 17β-E<sub>2</sub> supplementation on the protein expression levels of ECaC1 in rat kidneys. (A) Immunoperoxidase staining for ECaC1 in kidney cortex sections from OVX rats (top) and OVX/high-dose 17β-E<sub>2</sub>-treated rats (bottom). (B) Protein expression levels, calculated by counting the relative amounts of positive tubules in 10 to 15 views in each immunostained section. +E<sub>2</sub>L, supplemented with 2 × 32 μg 17β-E<sub>2</sub>/d; +E<sub>2</sub>M, supplemented with 2 × 125 μg 17β-E<sub>2</sub>/d; +E<sub>2</sub>H, supplemented with 2 × 500 μg 17β-E<sub>2</sub>/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-E<sub>2</sub>.

body weights (Table 3), compared with wild-type animals (>20 g) (14,19). After 17β-E<sub>2</sub> treatment, no significant change in body weight was observed. The 1α-OHase-knockout mice exhibited severe hypocalcemia, with plasma Ca<sup>2+</sup> levels of

1.63 ± 0.06 mM. Surprisingly, after treatment with 17β-E<sub>2</sub>, plasma Ca<sup>2+</sup> levels significantly increased to 2.03 ± 0.12 mM. Measurement of serum 17β-E<sub>2</sub> levels confirmed the increase in 17β-E<sub>2</sub> levels after treatment (Table 3).

Table 3. Effects of  $17\beta\text{-E}_2$  in  $1\alpha\text{-OHase}$ -knockout mice<sup>a</sup>

	Control (n = 4)	$17\beta\text{-E}_2$ (n = 4)
Body weight (g)		
beginning	15 ± 2	16 ± 1
final	15 ± 2	14 ± 1
Serum level		
$17\beta\text{-E}_2$ (pg/ml)	ND	67 ± 12
$\text{Ca}^{2+}$ (mM), before	1.54 ± 0.06	1.63 ± 0.06
$\text{Ca}^{2+}$ (mM), after	1.69 ± 0.10	2.03 ± 0.12 <sup>b</sup>

<sup>a</sup>  $1\alpha\text{-OHase}$ , 25-hydroxyvitamin  $\text{D}_3\text{-}1\alpha\text{-hydroxylase}$ ; control,  $1\alpha\text{-OHase}$ -knockout mice;  $17\beta\text{-E}_2$ ,  $1\alpha\text{-OHase}$ -knockout mice supplemented with 10  $\mu\text{g}$   $17\beta\text{-E}_2/\text{d}$ ; ND, not detectable. Data are presented as mean ± SEM.

<sup>b</sup>  $P < 0.05$  versus all (before and after).

### $1,25(\text{OH})_2\text{D}_3$ -Independent Effect of $17\beta\text{-E}_2$ on $\text{ECaC1}$ Expression

To investigate the involvement of  $1,25(\text{OH})_2\text{D}_3$  in the stimulatory effect of  $17\beta\text{-E}_2$  supplementation on the expression of  $\text{ECaC1}$  and other renal  $\text{Ca}^{2+}$ -transport proteins, control and  $17\beta\text{-E}_2$ -treated  $1\alpha\text{-OHase}$ -knockout mice were used. A 2.5-fold increase in  $\text{ECaC1}$  transcript levels in the kidney cortex of  $17\beta\text{-E}_2$ -treated mice, compared with control mice, was observed (Figure 3A). However, in contrast to the upregulation of  $\text{ECaC1}$ , no significant changes in the expression of calbindin- $\text{D}_{28\text{K}}$ ,  $\text{NCX1}$ , or  $\text{PMCA1b}$  were observed after treatment (Figure 3, B to E). Immunohistochemical analyses confirmed the increase in  $\text{ECaC1}$  expression, after  $17\beta\text{-E}_2$  treatment, at the protein level, as indicated by the increased staining in the kidney cortex of  $17\beta\text{-E}_2$ -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  $\text{ECaC1}$  protein after  $17\beta\text{-E}_2$  treatment (Figure 4B).

## Discussion

The main conclusion from this study is that estrogen upregulates the expression of  $\text{ECaC1}$  in the kidney, in a  $1,25(\text{OH})_2\text{D}_3$ -independent manner. By upregulating  $\text{ECaC1}$  expression, as the rate-limiting step in transcellular transport, estrogen could be positively involved in  $\text{Ca}^{2+}$  reabsorption. Estrogen deficiencies after menopause result in bone loss, which is associated with increases in plasma and urinary  $\text{Ca}^{2+}$  levels (4). It has been generally reported that the increases in plasma and urinary  $\text{Ca}^{2+}$  levels are secondary to increases in bone resorption. However, some studies have demonstrated that the increases in urinary  $\text{Ca}^{2+}$  levels after menopause are not attributable to increases in the filtered load, suggesting that estrogen also has an effect on renal  $\text{Ca}^{2+}$  handling (5,6). This study identified  $\text{ECaC1}$  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  $\text{ECaC1}$  mRNA and protein expression.  $\text{ECaC1}$  protein was observed

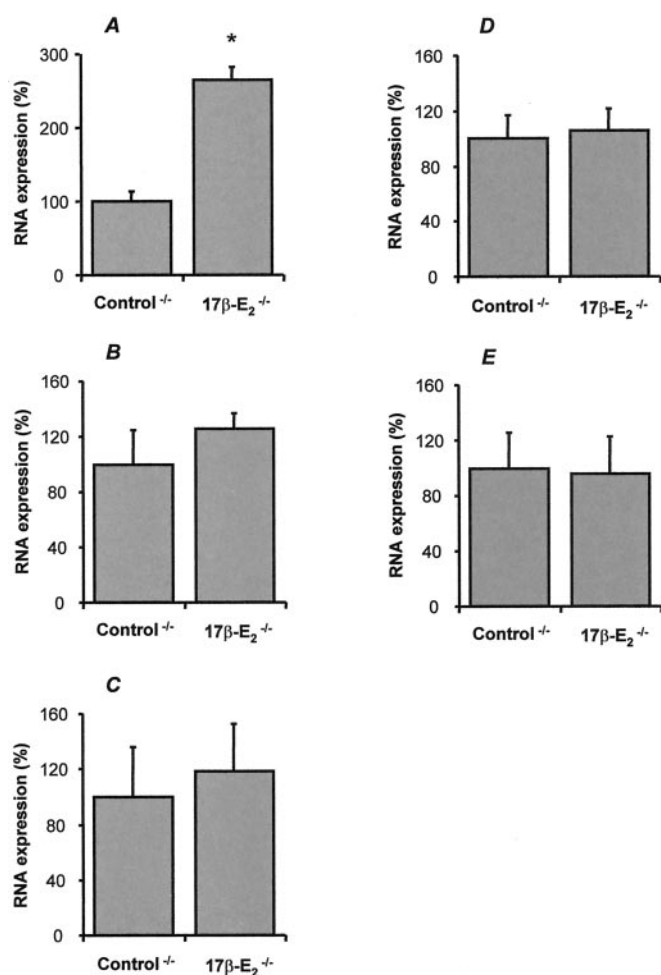
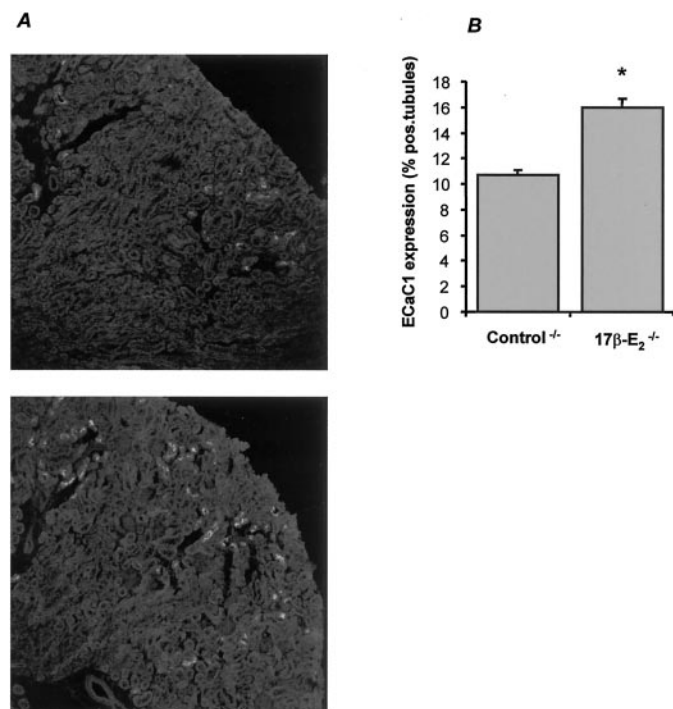


Figure 3. Effects of  $17\beta\text{-E}_2$  on the mRNA expression levels of  $\text{ECaC1}$  and other  $\text{Ca}^{2+}$ -transport proteins in kidneys from 25-hydroxyvitamin  $\text{D}_3\text{-}1\alpha\text{-hydroxylase}$  ( $1\alpha\text{-OHase}$ )-knockout mice. The mRNA expression levels of  $\text{ECaC1}$  (A), calbindin- $\text{D}_{28\text{K}}$  (B), calbindin- $\text{D}_{9\text{K}}$  (C),  $\text{PMCA1b}$  (D), and  $\text{NCX1}$  (E) were measured for the different experimental groups by using real-time quantitative PCR. Control<sup>-/-</sup>,  $1\alpha\text{-OHase}$ -knockout mice;  $17\beta\text{-E}_2$ <sup>-/-</sup>,  $1\alpha\text{-OHase}$ -knockout mice supplemented with 10  $\mu\text{g}$   $17\beta\text{-E}_2/\text{d}$ . Data are presented as means ± SEM (n = 4). \* $P < 0.001$  versus  $1\alpha\text{-OHase}$ -knockout mice.

exclusively at the apical side of distal tubular cells, in agreement with previous studies (17,20). The increased mRNA levels after  $17\beta\text{-E}_2$  treatment could be attributable to enhanced transcriptional activity or mRNA stabilization. In addition to increases in mRNA abundance, translational regulation of  $\text{ECaC1}$  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  $17\beta\text{-E}_2$  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  $\text{ECaC1}$  expression. Interestingly, Weber *et al.* (21) recently described an estrogen response element in the promoter se-



**Figure 4.** Effects of  $17\beta\text{-E}_2$  on the protein expression levels of ECaC1 in the kidneys of  $1\alpha\text{-OHase}$ -knockout mice. (A) Immunoperoxidase staining for ECaC1 in kidney cortex sections from control (top) and  $17\beta\text{-E}_2$ -supplemented (bottom)  $1\alpha\text{-OHase}$ -knockout mice. (B) Protein expression levels, calculated by counting the relative amounts of positive tubules in 10 to 15 views in each immunostained section. control<sup>-/-</sup>,  $1\alpha\text{-OHase}$ -knockout mice;  $17\beta\text{-E}_2$ <sup>-/-</sup>,  $1\alpha\text{-OHase}$ -knockout mice supplemented with  $10\ \mu\text{g}\ 17\beta\text{-E}_2/\text{d}$ . Data are presented as means  $\pm$  SEM ( $n = 4$ ). \* $P < 0.001$  versus  $1\alpha\text{-OHase}$ -knockout mice.

quence of the mouse ECaC2 gene; the response element was not present in the mouse ECaC1 gene. Alternatively, transcriptional activation by the estrogen-liganded ER can be mediated via activator protein 1 (AP-1) binding sites or GC-rich stimulatory protein (Sp1) binding sites (22,23). Importantly, the human ECaC1 promoter contains several of these AP-1 and Sp1 sites (20,24). Several of these AP-1 and Sp1 binding sites can be observed in the 5'-upstream region of the translation initiation site in the mouse ECaC1 gene and could be involved in the positive effect of  $17\beta\text{-E}_2$  treatment on ECaC1 mRNA expression. Future studies using ER-knockout mice should indicate whether the observed effects on ECaC1 expression are mediated via ER or an unknown mechanism. Furthermore, detailed promoter analysis is necessary to identify the regulatory sites involved in this estrogen-mediated regulation of ECaC1.

Because the activity of ECaC1 is tightly controlled by cytosolic  $\text{Ca}^{2+}$  concentrations, a sufficient cytosolic  $\text{Ca}^{2+}$ -buffering capacity is essential (25,26). This study demonstrates that, in rat kidney, ECaC1 and calbindin- $\text{D}_{28\text{K}}$  are synchronically upregulated, suggesting that this requirement is fulfilled. There is evidence that an estrogen-responsive promoter exists in the 5'-upstream region of the mouse calbindin- $\text{D}_{28\text{K}}$  gene

(27). Criddle *et al.* (28) demonstrated that estrogen treatment increased renal calbindin- $\text{D}_{28\text{K}}$  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  $\text{Ca}^{2+}$  extrusion across the basolateral membrane is mediated primarily by NCX1 (13,29). Taken together, these findings suggest a positive effect of  $17\beta\text{-E}_2$  on renal tubular reabsorption of  $\text{Ca}^{2+}$ . However,  $17\beta\text{-E}_2$  treatment resulted in decreased serum  $\text{Ca}^{2+}$  levels. This effect of estrogen therapy on serum  $\text{Ca}^{2+}$  levels was previously observed among human subjects (30). This apparent discrepancy can be explained on the basis of the increased  $\text{Ca}^{2+}$  requirements for estrogen-deficient animals. Correction of estrogen deficiencies results in decreased bone resorption and increased formation, causing slight decreases in plasma  $\text{Ca}^{2+}$  concentrations (3).

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

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



required for optimal stimulation of  $\text{Ca}^{2+}$  transport, eventually leading to normalized  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels. This is in accord with previous studies that suggested that ECaC1 represents the rate-limiting step in active  $\text{Ca}^{2+}$  transport (12,13). Also, the intestine must be considered to contribute to the increase in serum  $\text{Ca}^{2+}$  levels. It was previously demonstrated that estrogen has a positive effect on intestinal  $\text{Ca}^{2+}$  absorption, and it has been suggested that this effect is independent of  $1,25(\text{OH})_2\text{D}_3$  (16,38,39). ECaC1 and its homolog ECaC2 are present in the duodenum, as are other transport proteins necessary for active  $\text{Ca}^{2+}$  transport (11,17).

In addition to direct effects on bone, estrogen deficiencies might directly affect renal and intestinal  $\text{Ca}^{2+}$  handling, resulting in a continuing negative  $\text{Ca}^{2+}$  balance. Furthermore, interactions with other calciotropic 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  $17\beta\text{-E}_2$  is positively involved in renal  $\text{Ca}^{2+}$  reabsorption via the upregulation of ECaC1, an effect independent of  $1,25(\text{OH})_2\text{D}_3$ . 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.

## Acknowledgments

We thank Organon Nederland BV for kindly providing kidney tissue from the OVX rat model study. This work was supported by grants from the Dutch Organization of Scientific Research (Grants Zon-Mw 902.18.298 and Zon-Mw 016.006.001) and by Shriners of North America. Dr. St. Arnaud is a Chercheur-Boursier of the Fonds de la Recherche en Santé du Québec.

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