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Evidence for Involvement of 17β-Estradiol in Intestinal Calcium Absorption Independent of 1,25-Dihydroxyvitamin D_3 Level in the Rat

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ABSTRACT

The sex steroid 17β-estradiol (17β-E_2) has a broad range of actions, including effects on calcium and bone metabolism. This study with 3-month-old Brown Norway rats was designed to investigate the role of 17β-E_2 in the regulation of calcium homeostasis. Rats were divided in four groups, sham-operated, ovariectomized (OVX), and OVX supplemented with either a 0.025-mg or 0.05-mg 17β-E_2 pellet implanted subcutaneously. After 4 weeks, in none of the groups was serum calcium, phosphate, or parathyroid hormone altered compared with the sham group, while only in the OVX rats was a significant reduction in urinary calcium found. Bone mineral density and osteocalcin were modified, as can be expected after OVX and 17β-E_2 supplementation. OVX resulted in a non-significant increase in serum 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). Supplementation with either one of the 17β-E_2 dosages resulted in an 80% reduction of 1,25$(OH)_2D_3$ and only a 20% reduction in 25-hydroxyvitamin D_3 levels. OVX, as well as supplementation with 17β-E_2, did not affect serum levels of vitamin D binding protein. As a consequence, the estimated free 1,25$(OH)_2D_3$ levels were also significantly decreased in the 17β-E_2–supplemented group compared with the sham and OVX groups. Next, the consequences for intestinal calcium absorption were analyzed by the in situ intestinal loop technique. Although the 1,25$(OH)_2D_3$ serum level was increased, OVX resulted in a significant decrease in intestinal calcium absorption in the duodenum. Despite the strongly reduced 1,25$(OH)_2D_3$ levels (18.1 ± 2.1 and 16.4 ± 2.2 pmol/l compared with 143.5 ± 29 pmol/l for the OVX group), the OVX-induced decrease in calcium absorption could partially be restored by supplementation with either 0.025 mg or 0.05 mg of 17β-E_2. None of the treatments resulted in a significant change in calcium handling in the jejunum, although the trends were similar as those observed in the duodenum. 17β-E_2 did not change the VDR levels in both the intestine and the kidney. In conclusion, the present study demonstrates that 17β-E_2 is positively involved in intestinal calcium absorption, and the data strengthen the assertion that 17β-E_2 exerts this effect independent of 1,25$(OH)_2D_3$. In general, 17β-E_2 not only affects bone turnover but also calcium homeostasis via an effect on intestinal calcium absorption. (J Bone Miner Res 1999;14:57–64)

INTRODUCTION

17β-ESTRADIOL (17β-E_2) has evolved from a steroid hormone involved in reproduction into a pleiotropic hormone. Its receptor has been identified in multiple tissues not considered classical targets, including the central nervous system, endocrine glands, skin, myocardium, aorta, kidney, intestine, and bone.[1–3] Therefore, 17β-E_2 should be considered to be more than a sex steroid.

The significance of estradiol for bone is perfectly illus-

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trated by the drop in bone mineral density (BMD) after the menopause and the low bone mass observed in the estrogen receptor as well as the aromatase-deficient individuals.[4,5] During growth (modeling bone), estradiol is involved in closure of the growth plate. In remodeling bone, estradiol is involved in the maintenance of the balance between bone resorption and bone formation,[6,7] thereby affecting BMD and bone architecture.[8–11] These effects can be direct and/or indirect via regulation of and/or interaction with other factors influencing bone metabolism, e.g., parathyroid hormone (PTH) and insulin-like growth factor I.[12–17]

Menopause is associated with decreased calcium absorption,[18] which can be normalized by 17β-E_2 supplementation.[19,20] A positive regulation of intestinal calcium absorption by estradiol has been suggested by Arjmandi et al.[21] Therefore, modulation of intestinal calcium absorption could be a target for estradiol and selective estrogen receptor modulators in postmenopausal osteoporosis. However, a study by Hope et al. demonstrated an increase in intestinal calcium transport in rats after ovariectomy (OVX).[22] So far, data on direct measurements of calcium movements across the duodenal mucosa in relation to estradiol status are lacking.

The present study was designed to provide knowledge on the role of estradiol in intestinal calcium absorption and bone metabolism. This was examined by using OVX rats supplemented with estradiol or not. The effect on calcium absorption was measured with the in situ loop technique.[23] In addition, the relationship of estradiol with the classical calciotropic hormones, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and PTH and the effects on BMD and renal calcium handling were assessed.

MATERIALS AND METHODS

Animals

Experiment 1: Thirty female Brown Norway rats were divided into four groups to evaluate effects of 17β-E_2 on intestinal calcium transport and calcium and bone metabolism parameters. Eight rats were sham operated (sham group) and 22 rats were ovariectomized (OVX group). In six OVX rats, a 0.025-mg 17β-E_2 60-day release pellet and in eight OVX rats a 0.05-mg 17β-E_2 pellet (Innovative Research of America, Toledo, OH, U.S.A.) was subcutaneously implanted in the neck. These pellets are formulated to release a constant amount of 17β-E_2 for up to 60 days when implanted subcutaneously.

Experiment 2: Thirty-six rats were equally divided into three groups of 12 rats each to study the effects of 17β-E_2 on intestinal and renal vitamin D receptor (VDR) and calbindin 28K content. In addition, effects on vitamin D–binding protein (DBP) and BMD were examined. Rats were sham operated, ovariectomized, or ovariectomized with implantation of a 0.05-mg 17β-E_2 pellet.

In both experiments, deionized water and a standard diet containing 0.7% calcium, 0.5% phosphorus, and 1.5 U of vitamin D_3/g (Hopefarm, Woerden, The Netherlands) were available ad libitum to the sham-operated up. The treated groups (OVX ± 17β-E_2) were fed the same amount of food per gram of body weight as the sham-operated group to minimize differences in body weight.

After 3 weeks, the rats were put in metabolic cages and overnight nonfasting urine was collected. After 4 weeks, all rats were bled under ether anesthesia from the abdominal aorta, and several parameters for calcium and bone metabolism were measured. In the second experiment, intestines and kidneys were collected to quantitate calbindin 28K and/or VDR content, and in serum, DBP was measured. The study was approved by the committee for use of laboratory animals of the Erasmus University, Rotterdam.

Analytical procedures

Serum calcium, inorganic phosphate, alkaline phosphatase, total protein, urinary calcium, inorganic phosphate, and creatinine were analyzed with standard methods. Osteocalcin was determined by a rat-specific radioimmunoassay (RIA).[24] Intact PTH was measured using a rat-specific immunoradiometric method (Nichols Institute, San Clemente, CA, U.S.A.). $1,25(OH)_2D_3$ was measured by immunoextraction followed by quantitation by ^{125}I-RIA (IDS, Boldon, U.K.). Concentrations of 25-hydroxyvitamin D_3 ($25(OH)D_3$) were measured by RIA (Incstar Corp., Stillwater, MN, U.S.A.). Serum concentrations of DBP were measured by radial immunodiffusion.[25] The ratio ($\times 10^3$) of the molar concentrations of $1,25(OH)_2D_3$ and DBP was used as an estimate of free hormone concentration.[26] Serum 17β-E_2 was measured by an extraction procedure using diethyl ether followed by RIA (DPC, Los Angeles, CA, U.S.A.). The BMD of the right femur was measured ex vivo using dual-energy X-ray absorptiometry (DPX-L; Lunar Corp., Madison, WI, U.S.A.). Femora were placed under 2.5 cm of water using the high-resolution mode, as recommended by the manufacturer. The Lunar Small Animal Software Package Ic was used for analysis. The coefficient of variation, evaluated by scanning the same femur 10 times over a short period of time, was 1.1%.

Calcium transport measurement

$^{45}CaCl_2$ (1.61 mCi/mg of Ca) was obtained from Amersham (Arlington Heights, IL, U.S.A.) and [1,2-^{3}H] polyethyleneglycol (PEG; MW 4000, 0.45 mCi/g) was obtained from DuPont-NEN (Boston, MA, U.S.A.).

Four weeks after operation, calcium transport measurements were performed under ether anesthesia by the in situ loop technique after overnight fasting.[23] Shortly thereafter, a midline incision was made in the bile duct and two intestinal segments were ligated. The first segment (duodenum) was from 1 cm distal to the pylorus to 2 cm distal to Treitz's ligament. The second part encompassed jejunum and was from 13–23 cm distal to the pylorus. Transport medium (750 μl), prewarmed to 37°C, was injected with a 25-gauge needle in each ligated segment. The transport medium contained 164 mM NaCl, 0.88 mM $^{45}CaCl_2$ (610–680 dpm/nmol), and [1,2-^{3}H]PEG (720 dpm/nmol). Following an equilibration period of 5 minutes, a 50-μl sample from

TABLE 1. EFFECTS OF OVX AND 0.025 MG 17β-ESTRADIOL (OVX + E_2L) OR 0.05 MG 17β-ESTRADIOL (OVX + E_2H) IN RATS 4 WEEKS AFTER OPERATION

	SHAM (n = 8)	*OVX* (n = 8)	*OVX + E_2L* (n = 6)	*OVX + E_2H* (n = 8)
Body weight				
beginning (g)	139 ± 3.9	140 ± 3.4	142 ± 1.4	140 ± 2.1
final (g)	138 ± 4.0	143 ± 3.3	135 ± 2.1	131 ± 2.7*
Organ weight				
uterus (g)	0.327 ± 0.036	0.082 ± 0.005†	0.601 ± 0.028†‡	0.617 ± 0.027†‡
spleen	0.358 ± 0.01	0.407 ± 0.01	ND	0.309 ± 0.01
Serum				
17β-E_2 (pmol/l)	16.1 ± 4.6	3.0 ± 1.5†	21.2 ± 4.0*	41.5 ± 4.6†‡

Data are presented as means ± SEM; *$p < 0.01$ vs. OVX; †$p < 0.001$ vs. sham; ‡$p < 0.001$ vs. OVX; ND, not determined.

the luminal content was taken by means of a transmural puncture to calculate the initial intraluminal solvent volume, ^{45}Ca and ^{40}Ca content. Twice 10 μl of the sample was dissolved in 5 ml of scintillation fluid (Packard, Downers Grove, IL, U.S.A.), and ^{45}Ca and ^{3}H values were determined in a Packard 2650 Tricarb scintillation counter. ^{40}Ca was measured by a calcium kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). Duodenal and jejunal loops were removed after 25 and 30 minutes, respectively. The length was measured, and two samples of 20 μl were taken from the luminal content for determination of ^{45}Ca and ^{3}H radioactivity. Also, the ^{40}Ca concentration in two samples of 10 μl was determined in the luminal fluid. The lumen was flushed with 500 μl of NaCl 0.9% to determine the recovery of ^{3}H-PEG in order to assess whether transport medium had leaked from the intestinal lumen to the abdominal cavity. Intestinal calcium transport was calculated via the following formulas.

1. *Ca-influx* (nmol of Ca per cm of intestine per 30 minutes): defined as the unidirectional movement of calcium from intestinal lumen into tissue (mean value over the period studied)

$$\text{Ca-influx} = [V_i(^{45}Ca_i) - V_f(^{45}Ca_f)]/SA \times L$$

where V_i (initial volume in μl) refers to the injected solvent volume corrected for dilution by residual endogenous luminal fluid at t_0 on the basis of the change in PEG concentration in the previous 5 minutes. V_f (final volume in μl) refers to solvent volume left at the end of the absorption period (t_{30}). This parameter is calculated as the product of the ratio of PEG concentration at t_0 versus t_{30} and V_i. $^{45}Ca_i$ and $^{45}Ca_f$ (dpm per μl of intraluminal fluid) refer to initial and final ^{45}Ca concentration at t_0 and t_{30}, respectively. *SA* (dpm per nmol Ca) refers to the specific activity of Ca. *L* refers to the length of the ligated intestinal segment.

2. *Net Ca absorption* (nmol Ca per cm of intestine per 30 minutes): defined as the total amount of Ca absorbed from the intestinal lumen

$$\text{Net Ca abs} = {}^{40}Ca_iV_i - {}^{40}Ca_fV_f$$

where $^{40}Ca_i$ and $^{40}Ca_f$ (nmol per μl of intraluminal fluid) refer to the initial and final ^{40}Ca concentration.

3. *Ca-efflux* (nmol of Ca per cm of intestine per 30 minutes): defined as unidirectional movement of calcium that enters the intestinal lumen from the tissue:

$$\text{Ca-efflux} = \text{Ca-influx} - \text{Net Ca abs}$$

In the calculations, it is assumed that ^{45}Ca-efflux is negligible.

VDR analysis

Nuclear extracts were prepared as described previously.[27] Briefly, 20 cm of proximal small intestines was excised and flushed with 20 ml of ice-cold TED-saline (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml TPCK, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Scraped mucosa and kidneys were washed three times in TED-saline for 10 minutes at 3000*g*. Mucosa and kidneys were homogenized in 10 vol (w/v) KTED-300 (TED, 300 mM KCl) in a glass-Teflon homogenizer and polytron, respectively. Homogenates were centrifuged for 1 h at 100,000*g*, and extracts were stored at −80°C. The concentration of VDR was measured by a VDR-specific ELISA.[28]

Calbindin 28K quantitation

Kidneys were homogenized (20% w/v) in phosphate-buffered saline in the presence of enzyme inhibitors (10^{-4} M PMSF, 10^{-4} M TPCK) using a polytron. Homogenates were centrifuged at 38,000*g*. Supernatant solution was stored at −80°C and analyzed as described previously.[29]

Statistical analysis

Data analysis involved estimation of the means, SDs, standard errors, and one-way analysis of variance. $p < 0.05$ was considered statistically significant.

RESULTS

General measurements

Table 1 shows that OVX and 17β-E_2 suppletion resulted in the anticipated phenotypes. OVX rats had reduced

TABLE 2. EFFECTS OF OVX AND 0.025 MG 17β-ESTRADIOL (OVX + E_2L) OR 0.05 MG 17β-ESTRADIOL (OVX + E_2H) ON CALCIUM AND BONE METABOLISM PARAMETERS 4 WEEKS AFTER OPERATION

	Sham (n = 8)*	*OVX* (n = 8)*	*OVX + E_2L* (n = 6)*	*OVX + E_2H* (n = 8)*
Serum				
calcium (mmol/l)	2.11 ± 0.03	2.15 ± 0.07	2.17 ± 0.27	2.18 ± 0.02
phosphate (mmol/l)	2.99 ± 0.07	3.18 ± 0.14	2.67 ± 0.13†	2.65 ± 0.06†
alkaline phosphatase (U/ml)	42.6 ± 1.4	47.7 ± 2.8	32.8 ± 1.7‡§	33.4 ± 1.0‡§
osteocalcin (μg/l)	514.4 ± 37.8	739.8 ± 59.9$^{\|}$	473.7 ± 26.5¶	392.3 ± 16.1$^{\S\#}$
25(OH)D_3 (nmol/l)	72.7 ± 2.5	71.3 ± 1.9	56.0 ± 2.8‡§	58.9 ± 2.5‡§
1,25(OH)$_2D_3$ (pmol/l)	105.4 ± 21.5	143.5 ± 29.0	18.1 ± 2.1‡§	16.4 ± 2.2‡§
PTH (ng/l)	41.9 ± 11.2	53.1 ± 12.2	58.5 ± 10.8	56.6 ± 6.9
Urine				
calcium/creatinine	0.80 ± 0.15	0.48 ± 0.05$^{\#}$	0.73 ± 0.03¶	0.98 ± 0.06§
BMD (g/cm^2)	0.183 ± 0.022	0.174 ± 0.012	ND	0.195 ± 0.026

Urinary calcium excretion was measured 3 weeks after operation.

* Except for BMD measurements, $n = 12$/group.

Data are presented as means ± SEM. $^{\dagger}p < 0.05$ vs. OVX; $^{\ddagger}p < 0.001$ vs. sham; $^{\S}p < 0.001$ vs. OVX; $^{\|}p < 0.01$ vs. sham; $^{\P}p < 0.01$ vs. OVX; $^{\#}p < 0.05$ vs. sham.

uterus weights and increased spleen weight. Although serum 17β-E_2 levels in OVX + 0.025 mg 17β-E_2 rats were similar as in sham-operated rats, uterus weights were significantly increased. Supplementation with the highest 17β-E_2 dose resulted in a similar increase in uterus weight as in the OVX + 0.025 mg 17β-E_2 group, while serum 17β-E_2 levels in the OVX + 0.05 mg 17β-E_2 rats were higher, i.e., at estrus level. Body weights in the treated groups were not significantly different from the sham-operated rats, but in the E_2H group they were significantly lower compared with the OVX rats.

Calcium and bone metabolism

No significant differences between 17β-E_2–treated groups and sham-operated rats in serum calcium and inorganic phosphate levels were detected, while serum inorganic phosphate levels were significantly lower compared with OVX rats.

OVX resulted in a significant reduction in BMD, while 17β-E_2 treatment restored BMD and even resulted in an increased BMD (Table 2). Effects of OVX and 17β-E_2 treatment on bone turnover are also reflected by changes in serum osteocalcin levels. In OVX rats, serum osteocalcin levels were elevated while treatment with 17β-E_2 significantly suppressed this increase (Table 2). Serum alkaline phosphatase levels were significantly reduced in both 17β-E_2–replete groups compared with sham-operated as well as OVX rats (Table 2).

Vitamin D

Serum total calcitriol levels were not significantly elevated (by about 30%) in the OVX rats. In the 17β-E_2–replete rats, serum total 1,25(OH)$_2D_3$ levels were significantly decreased by more than 80% in comparison with sham-operated rats. Serum 25(OH)D_3 levels were only reduced by about 20% in 17β-E_2–replete rats, while serum PTH levels were similar in all groups (Table 2). To address whether the major changes observed in total 1,25(OH)$_2D_3$ are reflected in the free levels of 1,25(OH)$_2D_3$, in the second experiment the DBP levels were determined. As can be seen in Table 3, the serum DBP levels were not affected by OVX or 17β-E_2 treatment. As a consequence, also the free 1,25(OH)$_2D_3$ levels are strongly reduced after OVX with 17β-E_2 suppletion.

Calcium transport

Despite normal or even somewhat raised serum 1,25(OH)$_2D_3$ levels in OVX rats, calcium influx in the duodenum was significantly reduced by 45% compared with the sham-group (Fig. 1A). Calcium efflux was increased, although not significantly, by 41% in the OVX group. These opposite changes in calcium influx and calcium efflux resulted in a significantly decreased net calcium absorption in duodenum following OVX (Fig. 1A).

The calcium influx was significantly reduced in both 17β-E_2–replete groups compared with sham-operated rats (Fig. 1). Although the 17β-E_2–replete rats had an ~90% lower serum 1,25(OH)$_2D_3$ level than OVX rats, calcium influx was even somewhat higher than in OVX rats. The calcium efflux was not changed in the 17β-E_2–replete rats compared with the sham group, and therefore calcium efflux was less than in the OVX group. Taken together, 17β-E_2 treatment partially restored net calcium absorption and was not significantly different from the sham group, despite a strongly reduced 1,25(OH)$_2D_3$ concentration (Fig. 1A).

In the jejunum, no significant differences between the groups were observed (Fig. 1B). However, similar trends were observed as in the duodenum. Again in 17β-E_2–replete rats, despite low serum 1,25(OH)$_2D_3$ levels, calcium influx was higher and calcium efflux was lower, resulting in an apparently higher net calcium absorption compared with OVX rats.

Overnight nonfasting urinary calcium excretion, which

TABLE 3. EFFECTS OF OVX AND 0.05 MG 17β-ESTRADIOL (OVX + E_2H) ON VITAMIN D BINDING PROTEIN, INTESTINAL VDR, AND RENAL VDR AND CALBINDIN 28K CONTENT 4 WEEKS AFTER OPERATION

	Sham (n = *12*)	*OVX* (n = *12*)	*OVX + E_2H* (n = *12*)
$1,25(OH)_2D_3$ (pmol/l)	87.6 ± 15.8	111.7 ± 13.9	28.9 ± 7.9*
DBP (μmol/l)	7.3 ± 0.1	7.7 ± 0.1	7.3 ± 0.1
Free $1,25(OH)_2D_3$ (index × 10^{-3})	12.3 ± 2.3	14.6 ± 1.9	4.0 ± 1.2*
Intestine			
VDR (fmol/mg protein)	190.9 ± 48.0	193.9 ± 32.1	217.3 ± 38.7
Kidneys			
VDR (fmol/mg protein)	18.2 ± 2.5	19.6 ± 2.2	22.2 ± 8.1
CaBP28K (μg/mg protein)	5.16 ± 0.25	5.07 ± 0.21	5.53 ± 0.19

Data are presented as means ± SE. *$p < 0.01$ vs. sham.

indirectly reflects total intestinal calcium absorption, was significantly lower in OVX rats compared with sham-operated and 17β-E_2–replete rats. No significant differences in urinary calcium excretion were found between 17β-E_2–replete and sham-operated rats (Table 2).

Intestinal VDR and renal VDR and calbindin 28K content

A possible mechanism by which 17β-E_2 affects intestinal calcium absorption is by facilitating $1,25(OH)_2D_3$ action via an increase in its receptor number, thereby potentially compensating for the reduction in $1,25(OH)_2D_3$ levels. Therefore, nuclear extracts of intestines of sham-operated, OVX and OVX + 0.05 mg 17β-E_2–replete rats were prepared and the VDR content was established. Neither OVX nor 17β-E_2 treatment resulted in a significant change in intestinal VDR number (Table 3). In addition, in the kidney, the VDR number was not different among sham, OVX, and 17β-E_2–replete rats (Table 3). In addition, we measured in the kidneys whether the alterations in renal calcium excretion could be related to changes in calbindin 28K levels. As shown in Table 3, neither OVX nor 17β-E_2 treatment modified the renal calbindin 28K levels.

DISCUSSION

This study shows that 17β-E_2 has important effects on intestinal calcium absorption. This conclusion is based on the following observations. First, in the present study, calcium influx from the duodenum of OVX rats was strongly reduced, although serum $1,25(OH)_2D_3$ levels were even slightly (about 30%) higher than in the sham rats. Although not significant, calcium efflux was even higher in 17β-E_2–deficient rats, and therefore net calcium absorption was significantly reduced in OVX rats. This supports previous findings that menopause is associated with decreased intestinal calcium absorption.[18] Furthermore, it is consistent with the observation that in postmenopausal women estrogen supplementation normalizes intestinal calcium transport.[20,30] Second, in both 17β-E_2–replete groups, serum $1,25(OH)_2D_3$ levels were reduced by ~90% compared with the OVX group, which would have implicated an even further decrease in intestinal calcium transport.[31,32] However, in contrast to the OVX group, in both 17β-E_2–replete groups, net calcium absorption in the duodenum was not significantly different from the sham group. These direct calcium movement measurements support the calcium absorption data derived from food-intake studies.[21] Finally, an indirect measurement, nocturnal nonfasting urinary calcium excretion, which can be considered to reflect intestinal calcium absorption in rats eating during the night, was significantly higher in the 17β-E_2–replete groups compared with the OVX rats.

The effect of 17β-E_2 on intestinal calcium absorption can be exerted by modulating $1,25(OH)_2D_3$ action or independent of $1,25(OH)_2D_3$. The significantly reduced calcium absorption in the 17β-E_2–deficient OVX group, while the $1,25(OH)_2D_3$ levels are increased, suggest that in the absence of 17β-E_2 the intestine is desensitized for the action of $1,25(OH)_2D_3$. In other words, 17β-E_2 sensitizes the intestine for the action of $1,25(OH)_2D_3$. However, in case of a sensitizing action, this must be independent of changes in the VDR number in the intestine (Table 3). The calcium absorption in the 17β-E_2 supplemented groups, while $1,25(OH)_2D_3$ levels are strongly reduced (80–90% lower than in the sham and OVX groups, respectively) shows that 17β-E_2 has a direct effect on intestinal calcium absorption independent of $1,25(OH)_2D_3$ level. Our observations on the VDR are consistent with the recent data from Kinyamu et al., who found no change in intestinal VDR level, while intestinal calcium absorption was decreased in postmenopausal women compared with premenopausal women.[33] However, previously Ebeling et al. have reported that the decreased intestinal calcium absorption of postmenopausal women could be attributed to a decline in intestinal VDR number, but the number of elderly subjects in this study was small.[34] Although an interaction between 17β-E_2 and $1,25(OH)_2D_3$ can not be conclusively excluded, our data suggest a $1,25(OH)_2D_3$-independent effect of 17β-E_2 on intestinal calcium absorption. This is supported by the observations that 17β-E_2 stimulates calcium uptake in cultured rat duodenal enterocytes.[35] The 17β-E_2 effects on intestinal calcium absorption can be due to direct effects on the

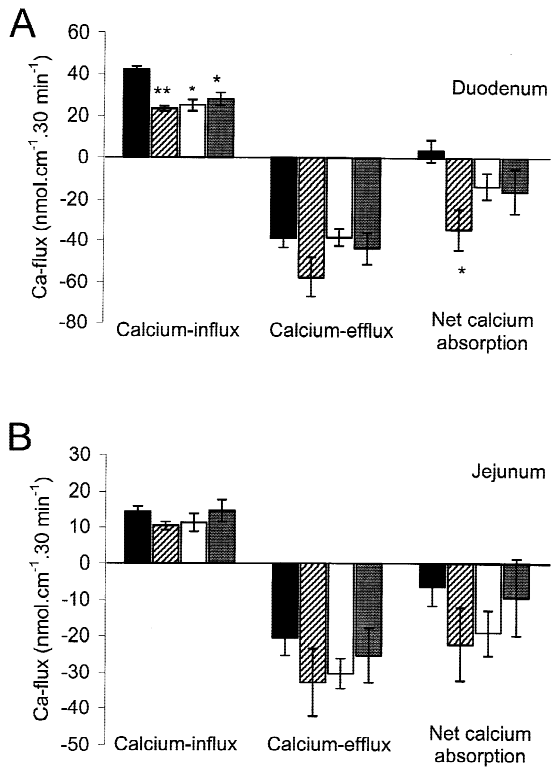

FIG. 1. Effects of OVX and OVX + 17β-E_2 on calcium flux in (A) duodenum and (B) jejunum. Calcium movements were measured and calculated as described in the Materials and Methods. Calcium influx is movement of calcium from the intestinal lumen into the animal; calcium efflux is movement of calcium from the animal into the intestinal lumen; net calcium absorption is difference between calcium influx and calcium-efflux. Solid bars = sham-operated rats (n = 8); hatched bars = ovariectomized rats (n = 8); open bars = OVX rats treated with 0.025 mg 17β-E_2 (n = 6); grey bars = OVX rats treated with 0.05 mg 17β-E_2 (n = 8). Data are presented means ± SE. *p < 0.05 and **p < 0.01 versus sham.

intestine. The estrogen receptor has been shown to be present in the intestine,[3] and, recently, the newly cloned estrogen receptor-β has been identified in the upper gastrointestinal tract.[36] The precise downstream mechanisms by which 17β-E_2 may affect intestinal calcium absorption are as yet purely speculative.

At kidney level, the reduction in 1,25$(OH)_2D_3$ levels after 17β-E_2 suppletion is not reflected by a change in renal calbindin 28K expression. Calbindin 28K is thought to play a role in calcium uptake from the lumen by renal epithelial cells,[37] and 1,25$(OH)_2D_3$ has been shown to regulate the expression of calbindin 28K.[38] Potentially, at kidney level, 17β-E_2 could compensate for the strong reduction in 1,25$(OH)_2D_3$ level independent of effects on VDR. It is more likely that the lack of changes in calbindin 28K is explained by the observation that the regulation of calbindin 28K gene expression may be more sensitive, under certain conditions, to changes in serum calcium levels than to changes in serum 1,25$(OH)_2D_3$ levels.[39–40] In our study, no differences were observed in serum calcium levels as well as urinary calcium excretion between the sham and 17β-E_2–replete groups.

A marked observation in the present study was made regarding the 1,25$(OH)_2D_3$ levels. Serum total 1,25$(OH)_2D_3$ levels were somewhat but not significantly elevated following OVX. This is in agreement with earlier observations in rats.[10,41] Nyomba et al. found increased total serum 1,25$(OH)_2D_3$ levels but normal free serum 1,25$(OH)_2D_3$ levels following OVX because DBP levels were also elevated.[42] In estrogen-deficient women, both decreased[43,44] and normal serum 1,25$(OH)_2D_3$ levels[20,45–47] have been reported. Major changes in serum 1,25$(OH)_2D_3$ levels were found with 17β-E_2 supplementation. This resulted in a strong reduction of both total and free serum 1,25$(OH)_2D_3$ levels. This observation is in line with a study by Kalu et al.[10] in rats but appears to be in contrast to the human situation. In women, transdermal estrogen administration has no effect on either serum DBP or serum 1,25$(OH)_2D_3$ levels[41,48,49] or even an increase with ethinyl estradiol was observed.[50] Therefore, our observations question whether the rat is a valid model for understanding the human (patho)physiology of menopause-related changes in vitamin D homeostasis. However, the observed changes in intestinal calcium absorption are in line with those observed in postmenopausal women.[18,20,30] Also, several arguments have been put forward by others that the OVX is a suitable model for postmenopausal osteoporosis.[51]

It is unlikely that the large changes in 1,25$(OH)_2D_3$ levels can be explained by the relatively small decrease in serum 25$(OH)D_3$ levels in the 17β-E_2–replete group. As a consequence, renal conversion of 25$(OH)D_3$ into 1,25$(OH)_2D_3$ by 1α-hydroxylase should have been reduced and/or the catabolism of 1,25$(OH)_2D_3$ and 25$(OH)D_3$ should have been increased. Because the estrogen receptor has been demonstrated in the kidney,[1,52] 17β-E_2 could directly inhibit 1α-hydroxylase activity and/or stimulate 24-hydroxylase activity. With cultured opossum kidney cells, a suppressive effect of 17β-E_2 on 1α-hydroxylase activity has been shown.[53] The present study is not conclusive as to whether in the rat 17β-E_2 inhibits 1α-hydroxylase activity and/or induces 24-hydroxylase activity.

17β-E_2 does not alter 1,25$(OH)_2D_3$ synthesis via a reduction of the potent stimulator of 1α-hydroxylase PTH (Table 2). However, 17β-E_2 may have acted at the kidney to inhibit PTH-stimulated 1,25$(OH)_2D_3$ synthesis. In the literature, conflicting data are presented concerning the effect of 17β-E_2 on serum PTH levels and 1α-hydroxylase activity. Stock et al. have shown that 17β-E_2 inhibits the PTH-stimulated intracellular cAMP accumulation in opossum kidney cells.[54] In laying hens, 17β-E_2 reduces PTH receptor binding affinity in calvaria and kidney.[55] However, estrogen substitution in postmenopausal women may result in either unchanged[19,49,54] or increased renal 1α-hydroxylase activity, either due to increased serum PTH levels[30] or an increased renal responsiveness to PTH.[56] Furthermore, Ash et al. have shown in a rat model that estrogens promote PTH-induced synthesis of calcitriol.[57]

Although the present data clearly demonstrate that 17β-

E_2 facilitates calcium absorption, the reduction in calcium absorption after OVX could not be completely overcome by 17β-E_2 supplementation. Various factors, including general anesthesia,[58] intraluminal pH[59] and glucose,[60,61] and bile salt concentration[62] are known to affect intestinal calcium transport. Therefore, calcium influx may have been reduced, while calcium efflux remained unaffected, resulting in a negative calcium transport in the treated groups. However, these factors are expected to have equal influence on calcium transport in the various groups. Therefore, it is unlikely that they account for the differences among the various groups. A more likely explanation is that 17β-E_2 also enhances calcium transport in other parts of the intestines. However, this seems not to be the case in the jejunum, because no significant differences in net calcium absorption among the various groups in this segment were found. Another possibility is the cecum, which has been shown to play an important role in calcium absorption in rats[63,64] and which expresses the estrogen receptor.[3] Another explanation could be that in addition to estrogens, other hormones or regulatory factors are reduced after OVX, which are not restored after estrogen replacement.

In conclusion, the present study demonstrates that 17β-E_2 is positively involved in intestinal calcium absorption. In addition, it provides results that 17β-E_2 may have a direct effect on intestinal calcium absorption independent of $1,25(OH)_2D_3$. The reduction in 17β-E_2 not only affects bone metabolism but also directly affects calcium homeostasis via effect on the intestine.

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