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# Estrogen Increases 1,25-Dihydroxyvitamin D Receptors Expression and Bioresponse in the Rat Duodenal Mucosa*

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**ABSTRACT**

Menopause and estrogen deficiency are associated with apparent intestinal resistance to vitamin D, which can be reversed by estrogen replacement. The *in vivo* influence of estrogens on duodenal vitamin D receptor (VDR) was studied in three groups of rats: ovariectomized (OVX), sham-operated, and ovariectomized rats treated daily with estrogen (40 $\mu$g/kg BW) for 2 weeks (OVX + E). Estrogen administration to OVX rats resulted in a 2-fold increase in VDR messenger RNA transcripts. 1,25$(OH)_2D_3$ was shown to bind specifically to one class of receptors in duodenal mucosal extracts, with a dissociation constant of 0.03 nM. Binding was significantly increased in duodenal extracts from OVX + E rats, compared with OVX rats (735 ± 81 *vs.* 295 ± 26 fmol/mg protein; $P < 0.001$); a comparable, 1.5- to 2-fold increase in VDR protein expression was observed in Western blot analyzes of the duodenal mucosa. Markers of VDR activity were increased in estrogen-exposed rats: calbindin-9k messenger RNA transcript content was 1.4- to 1.6-fold higher, and alkaline phosphatase activity was 1.4- to 3-fold higher in sham-operated and OVX + E, respectively, compared with OVX. 25(OH)D, 1,25$(OH)_2D$, or PTH levels were not altered by estrogen treatment. Cumulatively, these findings suggest that estrogen up-regulates VDR expression in the duodenal mucosa and concurrently increases the responsiveness to endogenous 1,25$(OH)_2D$. Modulation of intestinal VDR activity by estrogen, and subsequent influence on intestinal calcium absorption, could be one of the major protective mechanisms of estrogen against osteoporosis. (*Endocrinology* **140:** 280–285, 1999)

INTESTINAL calcium absorption takes place via two main mechanisms: passive diffusion, which occurs when luminal calcium is high; and active absorption, a complex and not fully understood process mediated by 1,25$(OH)_2D_3$, which predominates when luminal calcium is low (1). Under physiological circumstances, 1,25$(OH)_2D_3$ is primarily produced in the kidneys under the influence of PTH stimulation. PTH secretion, in turn, is dependent on extracellular free calcium concentration, as sensed by calcium-sensing-receptors located in parathyroid cells' membranes (2). Overall, the rate of active intestinal calcium absorption is determined by physiologic interactions between various components of the PTH-vitamin D-endocrine system organized in a multilevel negative feed-back loop structure.

Intestinal calcium absorption declines with age, both in humans (3, 4) and in rats (5). A widely held hypothesis suggests that the decrease in intestinal calcium absorption results from a sequence of events initiated by low estrogen levels, causing increased bone resorption; released calcium increases extracellular space calcium concentration, which suppresses PTH secretion, followed by a subsequent decrease in 1,25$(OH)_2D_3$ production and in 1,25$(OH)_2D_3$ plasma concentration, and finally results in decreased intestinal calcium absorption (6).

Nevertheless, there is evidence that estrogen may be more directly involved in determining intestinal calcium absorption. Estrogen receptors (7–10), as well as estrogen-receptor-associated proteins, pS2 antigen (11–13) and ER-D5 (14), have been consistently demonstrated in the mucosa along the alimentary tract, suggesting a specific physiological role for estrogen in the intestine. Menopause, postmenopausal osteoporosis (6), and the postovariectomy state (5) are associated with decreased circulating estrogen and a concomitant decrease in calcium absorption. Moreover, available data indicates that the decrease in the basal levels of 1,25$(OH)_2D_3$ could not solely account for the decrease in calcium absorption, suggesting that the intestine of elderly or ovariectomized women is resistant to 1,25$(OH)_2D_3$ (15, 16). In addition, estrogen administration was shown to effectively restore the normal responsiveness of the intestine to 1,25$(OH)_2D_3$ in ovariectomized premenopausal women (16) and in postmenopausal women (17, 18).

Previous studies indicated an age-related decrease in intestinal vitamin D receptor (VDR) (19, 20). The number of VDRs is a primary determinant of the biological response to 1,25$(OH)_2D_3$, as previously shown in osteoblastic cell lines (21, 22) and in association with VDR-gene polymorphism in human populations (23–25). We have previously shown that estrogen increases the number of VDRs in the osteoblast-like cell line ROS 17/2.8, and that the increase in VDR number is

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associated with an increased responsiveness of the cells to $1,25(OH)_2D_3$ (22). Others have shown that estrogen increases VDR messenger RNA (mRNA) transcript in osteoblasts (26–28). Estrogen-mediated increase in VDR expression was also noted in other tissues and cell types, such as uterus (29, 30) liver (31), and human breast cancer cells (32).

Modulation of VDR expression in the small intestine by estrogen could account for the relative resistance to $1,25(OH)_2D_3$ in the senescent and estrogen-deprived intestine and its correction during estrogen replacement. It could also account, at least in part, for the protective role of estrogen replacement against osteoporosis in estrogen-deficiency states. To this end, the present study was designed to investigate the interaction between estrogen, VDRs, and markers of vitamin D bioactivity in the rat duodenal mucosa.

## Materials and Methods

Chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. 1,25-dihydroxyvitamin $D_3$ was kindly provided as a gift by Hoffmann-La Roche (Basel, Switzerland). 1$\alpha$ 25-Dihydroxy [26,27-methyl-[$^3$H]-vitamin $D_3$ (180 Ci/mmol) was obtained from Amersham Laboratories, (Amersham, England). AMV reverse transcriptase, ribonuclease inhibitor, and random hexamer were obtained from Boehringer Mannheim (Mannheim, Germany). *Taq* polymerase was from Beit-Ha'emek Laboratories (Beit-Ha'emek, Israel). Oligonucleotides used for PCR primers were synthesized in the Biotechnology Weizmann Institute of Sciences (Rehovot, Israel). Monoclonal rat anti-VDR antibody for immunodetection by Western blot was purchased from Chemicon International Inc. (Temecula, CA). Monoclonal rat anti-VDR antibody has extensive cross-reactivity with all avian and mammalian VDR. The enhanced chemiluminescence kit was from Amersham, Buckinghamshire, UK.

### *Experimental protocol*

Charles-River outbred female rats (120–150 g) were divided randomly into three groups, were maintained in separate plastic cages at 12-h light, 12-h dark cycles and had free access to Purina chow. Rats were ovariectomized (OVX) or sham-operated (Sham) under light ether anesthesia. A subgroup of ovariectomized rats received 17$\beta$-estradiol (40 $\mu$g/kg BW daily, sc) for 14 days, starting 24 h after ovariectomy (OVX + E). Estrogen was dissolved in absolute alcohol and maintained at −20 C. Immediately before administration, aliquots from the batch solution were diluted to 10% ethanol in normal saline. OVX rats received 10% ethanol vehicle only.

At the end of the treatment period, all animals were killed by decapitation; duodeni and uteri were removed. The duodenal mucosa was washed with ice-cold saline solution, laid open, and gently scraped. Uteri were removed, cleaned, and weighed to confirm estrogenic effect.

### *Serum collection*

Blood was collected in chilled tubes for 25-hydroxyvitamin D [25-(OH)D], 1,25-dihydroxyvitamin D [1,25-$(OH)_2$D)], and PTH determinations. The serum was subsequently separated, in a refrigerated centrifuge, and kept at −20 C until further processing.

### *Serum 25(OH)D and 1,25(OH)$_2$D determination*

Two-milliliter serum samples were submitted to lipid extraction by equal volumes of acetonitrile. 25-(OH)D and 1,25-$(OH)_2$D fractions were separated by a Sep-Pak (Waters, Milford, MA) separation procedure using C-18 and silica columns (33). The 25-(OH)D fraction was submitted to a competitive protein-binding assay using diluted rachitic rat serum as the binding protein (34). Quantification of the 1,25-$(OH)_2$D fraction was achieved by a VDR-binding assay (35) using calf thymus VDR (Nichols Institute Diagnostics, San Juan Capistrano, CA) as the ligand binder. All determinations were carried out in triplicate. Results are mean ± SE and are expressed as ng/ml serum of 25-(OH)D, or as pg/ml serum of 1,25-$(OH)_2$D.

### *Serum PTH determination*

Serum PTH was measured with a rat-specific PTH RIA kit from Nichols Institute Diagnostics. Two hundred microliters of serum sample was mixed with 100 microliters of $^{125}$I-labeled rat PTH. A single PTH-antibody-coated bead was added and incubated at 4 C for 24 h. At the end of the incubation, the solution was discarded, and the beads were washed in phosphate-buffered solution (0.01 M), pH 7.4. The radioactivity was assessed in a $\gamma$ counter. PTH concentrations were determined with a standard curve from appropriate rat-PTH standards.

### *RNA isolation*

Total RNA was extracted from scraped duodenal mucosae, according to a protocol for single-step RNA isolation based on acid guanidinium-thiocyanate-phenol-chloroform extraction, using Tri-reagent solution (36). Aliquots of total RNA were separated in sterile tubes and quantified.

### *Northern blot procedures*

After denaturation, 35-$\mu$g total RNA samples were submitted to electrophoresis in 1% agarose, 2.2 M formaldehyde gel, transferred to nylon membranes (Hybond N+, Amersham, Buckinghamshire, UK), and hybridized with $^{32}$P-labeled VDR, calbindin-9k, and $\beta$-actin probes. The probes were labeled with $\alpha$ $^{32}$P-CTP by a random-primed DNA labeling procedure with Klenow polymerase (Random Primed-DNA Labeling Kit, Boehringer Mannheim Biochemica, Mannheim, Germany).

### *Probe preparation by PCR*

Complementary DNA sequences for the detection of mRNA transcripts of VDR, $\beta$-actin, and calbindin-9k genes by Northern blot procedures were obtained by RT-PCR of intestinal RNA extract, according to Iida *et al.* (37) using appropriate primers and reverse transcriptase enzyme. The PCR buffer consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxynucleotide triphosphates, and 1.25 U/50 $\mu$l *Taq* polymerase. The VDR-gene PCR primers consisted of bases 41–60 in the sense direction (5′-GTGACTTTGACCGGAACGTG-3′) and bases 301–320 in the antisense direction (5′-ATCATCTCCCTCT-TACGCTG-3′) of the VDR gene (37), and the calculated PCR product length was 280 bp. The PCR program involved 35 cycles: 40 sec at 94 C, 60 sec at 50 C, and 90 sec at 72 C.

The calbindin-9k primers corresponded to the reported complementary DNA (cDNA) sequence of the rat intestinal vitamin D-dependent calcium binding protein (38). They consisted of bases 1–21 in the sense direction (5′-GAGACCTCACCTGTTCCTGTC-3′) and bases 387–406 (5′-GTCTCGGAATTTGCTTTAT-3′) in the antisense direction. The calculated PCR product length was 406 bp. The PCR program involved 30 cycles: 60 sec at 94 C, 90 sec at 55 C, and 90 sec at 72 C.

The primers used to obtain the probe to the housekeeping gene $\beta$-actin corresponded to bases 2846–2863 in the sense direction (5′-TCCTAGCACCATGAAGATC-3′) and to bases 3140–3158 in the antisense direction (5′-AAACGCAGCTCAGTAACAG-3′). The PCR program used for these sets of primers involved 30 cycles: 40 sec at 94 C, 60 sec at 60 C, and 90 sec at 72 C. The calculated PCR product length was 190 bp (37).

In ensuing probe amplification PCR was performed using cDNA templates obtained by RT-PCR and appropriate primers for VDR, $\beta$-actin, and calbindin-9k genes, at the conditions described previously.

### *1,25(OH)$_2$D receptor (VDR) assay*

VDR protein expression was determined by a ligand-binding assay, as previously described (39), in 250 $\mu$l of the cells' 30,000 × *g*-soluble fraction after homogenate extraction with hypertonic buffer containing 0.3 M KCl. Parallel incubations of the soluble fractions were carried out with different concentrations of [$^3$H]-1,25$(OH)_2D_3$, between 20–160 fmol, with or without the addition of 100-fold molar concentration of the radio-inert (cold) 1,25$(OH)_2D_3$. $K_d$ (dissociation constant) and maximal

specific binding capacities (Bmax) were determined by Scatchard analysis.

### *Western blot analysis*

Intestinal tissue lysates were separated on a 10% SDS gel and electroblotted onto nitrocellulose gels. Immunodetection used mouse monoclonal antibodies raised against VDR. After incubation with the anti-VDR antibodies, the gels were treated with goat antirat Igs conjugated to horseradish peroxidase. Visualization of the VDR protein was attained with an enhanced chemiluminescence kit, according to the manufacturer's instructions.

### *Quantification of autoradiogram signals*

The signals of the Northern and Western autoradiograms were quantified using a densitometric scanner with the Fujix Phosphor-Imager apparatus and the BAS-1000 Bio-Imaging Analyzer (Fuji Photo Film Co., Ltd. Co., Japan). Results of the densitometric determinations of VDR and calbindin-9-kDa mRNA were corrected according to the corresponding $\beta$-actin gene-transcription.

### *Alkaline phosphatase activity in the colonic mucosa*

Cytosolic enzyme activity was determined in homogenates from freshly isolated duodenal scrapings, with p-nitrophenylphosphate as substrate, as previously described (40).

### *Protein determination*

Protein concentration in the different suspensions was determined by a Pierce kit (Pierce Chemical Co., Rockford, IL) (41), employing a microbicinchoninic acid-based protein assay and BSA as the protein standard.

### *Statistical analysis*

Statistical evaluation for two-group analysis was carried out by the unpaired Student's $t$ test. Results are expressed as mean ± SE.

## Results

Throughout this study, uterine weight served as an independent marker for estrogenic activity in the different groups of rats (Table 1). The markedly reduced uterine weight in OVX rats confirmed least exposure to estrogen in this group, compared with the Sham and OVX + E groups. The higher uterine weights of the OVX + E group are attributed to the supraphysiological dose of estradiol and to lack of the physiological cyclicity in estrogen production.

### *Effect of estradiol on VDR transcription*

Northern blot analysis for mRNA transcripts of VDR indicated that the strongest expression was achieved after estrogen treatment (Fig. 1). Expression of the VDR mRNA transcripts was also notable in Sham rats.

Densitometric quantification of the signals produced by the hybridization in each treatment group, normalized according to $\beta$-actin mRNA expression, indicated an increase of approximately 2-fold in VDR mRNA content in estrogen-exposed animals (Sham and OVX + E), compared with OVX alone (Fig. 2).

### *Effect of estradiol on VDR protein expression*

*1,25(OH)$_2$D$_3$ binding studies.* 1,25(OH)$_2$D$_3$ was shown to bind specifically to one class of receptors in hypertonic-salt extracts of duodenal mucosal extracts (Fig. 3 and Table 1). VDR concentration (maximal bound 1,25(OH)$_2$D$_3$ per mg protein; Bmax) from Sham and OVX + E rats was significantly (at least 2-fold) higher than that of duodenal extracts from OVX rats.

*Western-blot analysis.* Western-blot analysis detected a VDR immunoreactive protein of 48 kDa (Fig. 4). The intensity of the corresponding bands from Sham and OVX + E were markedly stronger than those obtained from OVX rats. Quantification of VDR expression indicated a 1.6- to 2-fold increase in VDR-protein expression in the estrogen-exposed rats, compared with the estrogen-deprived rats (Table 1).

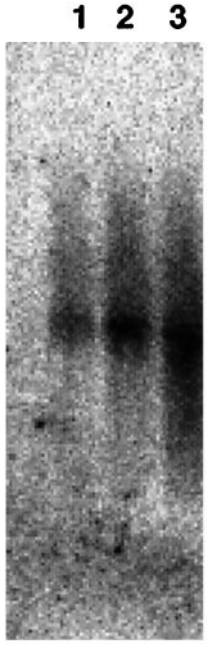


FIG. 1. Phosphor-Imager results of Northern blot analysis of RNA (35 $\mu$g/lane) from rat duodenal tissues probed with $^{32}$P-labeled rat VDR cDNA and exposed for 10 h. Lane 1, OVX; lane 2, Sham; lane 3, OVX + E. Rat VDR mRNA expression is most intense in duodenal tissue from OVX + E rats (lane 3).

**TABLE 1.** Effect of estrogen on rats' uterine weight, duodenal VDR protein expression by Western-blot, and duodenal VDR binding characteristics

| | OVX | Sham | OVX+E |
|---|---|---|---|
| Uteri weight (mg) | 99 ± 12 | 235 ± 28[a] | 352 ± 30[a] |
| VDR (Western) | 7587 ± 761 | 10654 ± 1078[a] | 14556 ± 1432[a] |
| $B_{max}$ (fmol/mg protein) | 295 ± 26 | 355 ± 52[a] | 735 ± 81[a] |
| $K_d$ (nM) | 0.031 ± 0.001 | 0.029 ± 0.005 | 0.032 ± 0.008 |

The 48-kDa VDR protein product bands, obtained by Western blotting, were densitometrically quantified.
[a] $P < 0.001$ vs. OVX. Values represent mean ± SD of 6 samples obtained from 6 different rats.

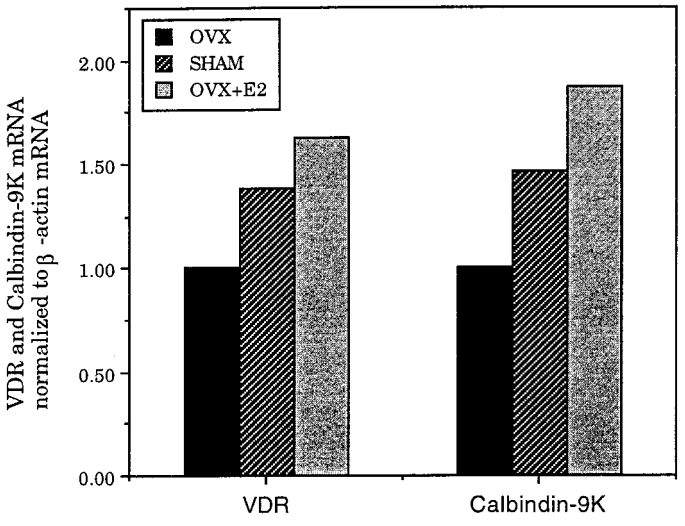


FIG. 2. Histogram of the effect of different treatments on rat VDR (Fig. 1) and calbindin-9k (Fig. 5) mRNA content in dodenal tissue from OVX, Sham, and OVX + E rats. The intensities of the transcripts were quantified by pixel densitometry and normalized to the respective intensities of the rat $\beta$-actin mRNA products obtained in the different treatment protocols.

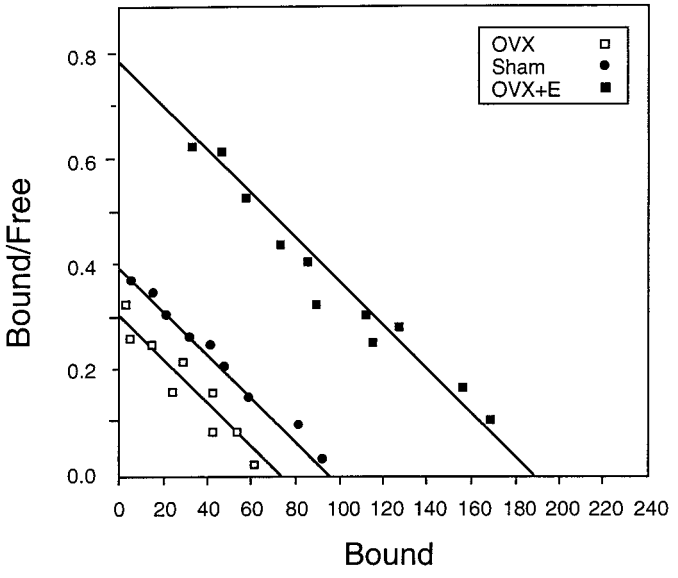


FIG. 3. Effect of estrogen on $1,25(OH)_2D_3$ binding to duodenal mucosal extracts. Representative Scatchard plots.

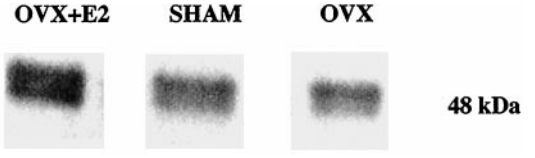


FIG. 4. Effect of estrogen on VDR protein expression, by Western-blot analysis in colonic mucosal extracts. Extracts from all treatment groups expressed the expected 48-kDa protein. See Table 2 for results of densitometric analysis of the corresponding bands.

*Effect of estradiol on functional expression of VDR in vivo*

*Duodenal alkaline phosphatase activity.* Alkaline phosphatase activity, a nonspecific vitamin D-induced marker of intestinal cell differentiation (42, 43), was determined in extracts of duodenal mucosa. The results indicate a significant increase in alkaline phosphatase activity in intestinal extracts from estrogen-exposed (Sham or OVX + E) rats, compared with estrogen-deprived (OVX) rats (Table 2).

*Northern blot analysis of calbindin-9k mRNA expression.* Calbindin-9k is a vitamin D-dependent calcium-binding protein specifically modulated by $1,25(OH)_2D_3$ at the genomic level (44–46). Using PCR and ethidium bromide staining, a single band of the expected size (406 bp) calbindin-9k product was detected (not shown). The purified cDNA fragment provided the probe for the subsequent Northern blot analysis. Northern blot band intensities of the transcribed calbindin-9k gene of duodenal extracts from Sham and OVX + E rats were markedly stronger, compared with OVX extracts (Fig. 5). Quantification of the signals obtained by each treatment was attained by densitometry and was corrected according to the corresponding $\beta$-actin signal for each sample. The results indicate a 1.6-fold increase in calbindin-9k mRNA content in estrogen-exposed, compared with estrogen-deprived, rats (Fig. 2).

*Serum PTH and vitamin D metabolites*

Estrogen treatment did not affect serum levels of either vitamin D metabolites or PTH (Table 3). Serum levels of PTH, 25-(OH)D and $1,25-(OH)_2D$ were similar in rats from all treatment groups.

## Discussion

These results reveal a direct relationship between estrogen exposure, VDR mRNA-transcript content, and VDR protein expression in the duodenal mucosa, suggesting that estrogen has a pivotal role in supporting VDR expression in the duodenal mucosa. Chan *et al.* (47) have previously documented a 30% decrease in intestinal VDR of OVX rats; however, those results could not be verified by a subsequent study (31).

Having documented that estrogen up-regulates VDR in the duodenal mucosa, we examined VDR-related bioresponses and observed a significant increase in colonic mucosal alkaline phosphatase activity and calbindin-9k steady-state mRNA content in duodenal mucosa of estrogen-exposed (compared with estrogen-deprived) rats. Alkaline phosphatase comprises a family of specific isoenzymes, the activity of which is associated with the differentiated phenotype of enterocytes and colonocytes (42, 43); calbindin-9k is one of the best documented biological markers of the hormonal action of $1,25-(OH)_2D_3$ at the genomic level (44–46). The present results indicate that, in addition to modulation of VDR expression, *in vivo* exposure to estrogen markedly enhances the response of markers of VDR activity in the duodenal mucosa to endogenous $1,25-(OH)_2D$. Neither serum $1,25-(OH)_2D$ nor PTH concentrations were altered by estrogen repletion; thus, we may conclude that the increase in intestinal VDR expression and activity was directly related to estrogen and is unlikely the result of an indirect effect of estrogen via PTH or $1,25-(OH)_2D$ on VDR expression (48, 49).

The present study, in addition to previous observations in osteoblasts (26–28, 50), indicates that estrogen increases total

**TABLE 2.** Effect of estradiol treatment on alkaline phosphatase activity in cytosolic extracts of rat duodenal mucosae

| | OVX | Sham | OVX+E |
|---|---|---|---|
| Alkaline phosphatase activity (U/mg protein/15 min) | 116 ± 12 | 157 ± 16[a] | 342 ± 14[b] |

[a] $P < 0.01$ *vs.* OVX.
[b] $P < 0.001$ *vs.* OVX.

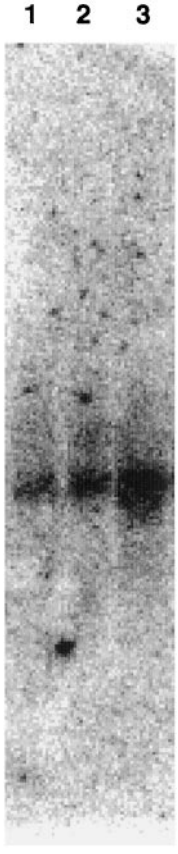


FIG. 5. Phosphor-Imager result of a Northern blot analysis of RNA (35 μg/lane) from rat duodenal tissues probed with $^{32}$P-labeled rat calbindin-9k cDNA and exposed for 10 h. Lane 1, OVX; lane 2, Sham; lane 3, OVX + $E_2$. Rat calbindin-9k mRNA expression is most intense in duodenal tissue from OVX + E rats.

VDR mRNA content. Theoretically, estrogen could exert its effect on a genomic level directly, by stimulating VDR gene transcription. However, an estrogen response element has not yet been discovered in association with the VDR gene (51, 52); it is thus possible that estrogen influences VDR gene transcription indirectly, through activation of other transcription factor(s) or through stabilization of the VDR mRNA transcript.

Available data indicates that the difference in VDR expression between young and adult rats is in the order of 1.5-fold in favor of young rats (19), comparable with the acute effect of ovariectomy on VDR expression observed in the present study, and with the effect of estrogen deprivation on intestinal calcium absorption (3, 4, 16, 17). Although some controversy still surrounds the issue of the role of VDR gene polymorphism in determining bone mineralization, recent observations indicate that the start-codon polymorphism,

**TABLE 3.** Effect of estradiol treatment on PTH and vitamin D metabolites

| | OVX | Sham | OVX+E |
|---|---|---|---|
| 25-(OH)D (ng/ml) | 42 ± 3 | 40 ± 5 | 45 ± 5 |
| 1,25$(OH)_2$D (pg/ml) | 55 ± 5 | 51 ± 3 | 48 ± 6 |
| PTH (pg/ml) | 28.2 ± 2.3 | 27.3 ± 4.4 | 28.9 ± 3.5 |

which is associated with differences in the VDR gene transcript level of a magnitude similar to that observed in the present study, is associated with significant differences in bone mineral density between carriers of the various polymorphic alleles (23–25). Taken together, these observations support the notion that changes in VDR expression, such as those observed in the present study, may be of physiological and pathophysiological significance. Our results suggest that estrogen deficiency directly influences VDR expression in the senescent intestinal mucosa, subsequently affecting intestinal calcium absorption. The effect of estrogen on intestinal VDR expression also provides a pertinent mechanism for the stimulatory effect of estrogen replacement on intestinal calcium absorption observed in estrogen-deficient rats and in women (53, 54).

Estrogen is one of the most effective available treatments for osteoporosis during menopause and in estrogen-deficiency states. The results of the present study, combined with previous observations from our group, indicate a modulatory effect of estrogen in osteoblastic cells (22), to suggest a unifying (although not exclusive) mechanism by which estrogen may protect the skeleton. Taken together, our previous observation in osteoblastic cells (22) and the present results, suggest that estrogen simultaneously increases VDR expression and specific 1,25-$(OH)_2$D-related activities in osteoblasts and in the intestinal mucosa, resulting in stimulation of bone-matrix protein synthesis, along with an increase in mineral supply. These complementary consequences of estrogen activity are likely the result of a similar molecular mechanism of estrogen activity in different types of cells, and they may account, at least in part, for the remarkable effectiveness of estrogen in maintaining bone integrity and in preventing osteoporosis.

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