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Estrogen Responsiveness of Renal Calbindin-D_{28k} Gene Expression in Rat Kidney

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Abstract In women, calcium excretion in the urine rises after menopause and falls with estrogen replacement therapy. The amount of calcium lost in the urine following estrogen therapy is less than should occur based on changes in serum calcium and the amount of calcium filtered by the kidney. This suggests there may be a direct effect of estrogen therapy to increase renal calcium reabsorption. Calbindin D_{28k} is a putative calcium ferry protein located in the distal renal tubules which has been shown to increase transcellular calcium transport. We proposed that estrogen loss after menopause may diminish gene expression of renal calbindin D_{28k} and subsequently diminish renal calcium reabsorption. We used the ovariectomized rat model of estrogen deficiency to investigate changes at the messenger RNA level of calbindin D_{28k} in ovariectomized rats (OVX), sham ovariectomized rats (S-OVX), and estrogen treated ovariectomized rats (E-OVX). We have demonstrated that ovariectomy in rats diminishes the gene expression of renal calbindin D_{28k}. The mRNA levels were approximately three times lower in OVX rats than S-OVX rats. Administration of 17β estradiol to OVX rats produced a significant increase in mRNA level to greater than the S-OVX rats by 4 h. Measurement of serum 1,25 dihydroxyvitamin D_3 showed lower levels in OVX rats than S-OVX rats but no significant change in E-OVX animals. In conclusion, our results indicate that estrogen increases renal calbindin D_{28k} mRNA levels, by a mechanism independent of changes in 1,25 dihydroxyvitamin D_3. This may result in increased expression of calbindin D_{28k} protein which may have a role in reducing renal calcium excretion. J. Cell. Biochem. 65:340–348. © 1997 Wiley-Liss, Inc.

Key words: estrogen; Calbindin D_{28k}; rat

The kidney is an important site for the regulation of calcium homeostasis, with the classical calcitropic hormones, parathyroid hormone (PTH), calcitonin, and 1,25 dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), exerting an influence on renal calcium reabsorption [Friedman and Gesek, 1993]. In women, estrogen deficiency as a result of menopause decreases bone mass and is a major factor in the development of osteoporosis and fracture in women. Estrogen must therefore play a central role in calcium homeostasis in women. In addition to its action on the skeleton, estrogen may also be involved in the regulation of renal calcium reabsorption and intestinal calcium reabsorption [Prince et al., 1991; Prince, 1994]. There is little data, however, on estrogen regulatory effects on renal calcium handling.

The renal calcium binding protein, calbindin, is a 28 kDa protein found in distal tubular cells, [Rhoten and Christakos, 1981] that may function as a transcellular calcium ferry protein [Friedman and Gesek, 1993; Johnson and Kumar, 1994]. As the distal tubule is the portion of the nephron considered to be important in hormonal regulation of renal calcium reabsorption [Friedman and Gesek, 1993] the distal tubular location of calbindin D28k suggests that it may participate in the fine tuning of calcium balance. A recent study [Bouhtiauy et al., 1994] has confirmed the ability of calbindin D_{28K} to increase calcium reabsorption by increasing calcium uptake into apical membrane vesicles from distal tubular cells, strongly suggesting a role for calbindin D_{28K} in renal calcium reabsorption.

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The expression of renal calbindin D_{28K} is known to be under the hormonal control of $1,25[OH]_2D_3$ [Li and Christakos, 1991; Gill and Christakos, 1993]. There is also evidence that an estrogen regulates calbindin expression in chick and mouse uterus [Nys et al., 1989, 1992; Opperman et al., 1992] and that estrogen responsive promoter exists in the five prime upstream region of the mouse calbindin D_{28k} gene [Gill and Christakos, 1995]. This suggests that multiple hormonal controls may exist for the expression of this protein.

We propose that estrogen loss after the menopause may diminish gene expression of renal calbindin D_{28K} and subsequently diminish renal calcium reabsorption. In order to study this, we used the ovariectomized rat model of estrogen deficiency [Goulding and Gold, 1989] to investigate changes at the messenger RNA level of calbindin D_{28K} in ovariectomized rats (OVX), sham ovariectomized rats (S-OVX), and estrogen treated ovariectomized rats (E-OVX).

MATERIALS AND METHODS

Animals and Experimental Design

All experiments were performed using 3-month-old, Sprague-Dawley rats which were obtained from the Animal Resources Centre (Murdoch, Western Australia) and kept under 12 h light-dark cycles. Ovariectomy and sham operations were performed under an intraperitoneal injection of 18 mg sodium pentobarbital. The sham operation was performed by exteriorizing the ovaries briefly. Successful ovariectomy was determined at the end of the experiment by confirming the absence of ovarian tissue and by examining the uterus, which is markedly atrophied as a result of estrogen deficiency. The study was carried out according to the Australian National Health and Medical Research Council guidelines for animal research and approved by the Animal Experimentation Ethics Committee of the University of Western Australia. After surgery all animals were fed a diet containing 0.8% calcium and 0.7% phosphate. For the time course experiments, animals were injected with estradiol valerate (20 µg) im 1 week after OVX. At varying times after estrogen injection, the animals were anaethetized with sodium pentobarbital and blood was collected by cardiac puncture for $1,25[OH]_2D_3$ measurement, or the kidneys were removed and immediately frozen in liquid nitrogen and then stored at -70°C prior to total RNA extraction. The dose response experiments were performed by injecting OVX rats with varying doses of estradiol valerate im 18 hours prior to the removal of the kidneys. All of the experiments were performed at least in duplicate.

Statistics

Data from the time course experiment was analyzed statistically using the SPSS for Windows program. Results are reported as mean ± SEM. The maximal densitometric response of calbindin-D28K mRNA is reported as the ratio with GAPDH mRNA. One way ANOVA and Duncan's multirange test was used to compare time points after estrogen administration and sham-OVX. A P value of less than 0.05 was considered significant by a two-tailed test.

cDNA Cloning

For rat calbindin D-28K cloning, total RNA from rat kidney was extracted with RNAzolB (Tel-Test, Texas) using the manufacturer's modification of the acid-guanidinium-thiocyanate-phenol-chloroform procedure [Chomczynski and Sacchi, 1987]. Single stranded DNA was generated by incubating total RNA with avian myeloblastosis virus reverse transcriptase (AMV-RT) in the presence of oligo dT (10 ng/µl), 1 mM dNTPs, 8 mM $MgCl_2$, and ribonuclease inhibitor (RNasin, Promega). A 780 base pair fragment of calbindin D-28K was synthesized using the published rat sequence sense primer 5' GGCAGAATCCCACCTGCAGT and anti-sense primer 5' GTTGTCCCCAGCAGAGAGAA [Lomri et al., 1989]. The PCR mixture contained single stranded DNA, 10 pmol of each primer and 0.2 mM of each dNTP in 50 mM KCl, 10 mM Tris.HCl (pH 9.0), 2.0 mM $MgCl_2$, 0.1% Triton X-100, and 1–5 units of *thermus aquaticus* (Taq) DNA polymerase. Twenty five cycles of 94°C (1 min), 55°C (1 min), and 72°C (1 min) were performed. The fragment of calbindin D-28K cDNA was then subcloned into the plasmid vector pGEM1 (Promega, Sydney). The nucleotide sequence of the cDNA insert was determined by the Sanger method of dideoxy-mediated chain termination using Sequenase (United States Biochemical, Ohio).

Rat GAPDH cDNA was a 1.1 kb insert containing sequences which were close to the

COOH terminus [Piechaczyk et al., 1984]. The Pst1 fragment initially cloned into pBR322 vector was subsequently subcloned into pGEM57f(+) according to the manufacturer's instructions.

RNA Analysis

Tissues were placed in sterile containers and snap frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted as described above, denatured, electrophoresed on 1% agarose/formaldehyde gels, and transferred to nylon membranes (Hybond N^+, Amersham). RNA was fixed to the membranes by UV irradiation and alkali fixation. Membranes were pre-hybridized at 55°C in hybridization solution (50% formamide, 0.9M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, 5x Denhardt's, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA) for at least 4 h. Hybridization was performed with [-^{32}P]UTP (Bresatec) labelled antisense RNA or [-^{32}P]dATP (Bresatec) labelled cDNA probes for 12–16 h at 55°C. Blots were washed once in 2x SSC; 0.1% SDS, and twice in 0.1x SSC; 0.1% SDS at 75°C for 30 min each where 20x SSC is 3 M NaCl, 0.3 M Na_3citrate (pH 7.4). Autoradiographic film (Cronex, Dupont) was exposed at −80°C.

In Situ Hybridization

In situ hybridization was used to localize the calbindin $D28_K$ gene transcripts in kidney as previously described [Jeffrey et al., 1994]. Briefly, 3-month-old rats (n = 2) were anaesthetized and perfusion fixed through the left ventricle with 150 ml of heparinized saline followed by 100 ml of fresh 4% paraformaldehyde in phosphate buffered saline (PBS). The kidneys and other tissues were excised and immersion fixed in neutral buffered formaldehyde. The tissue was then dehydrated, orientated in cross-section, and embedded in wax. Five µm sections were cut, dewaxed, and rehydrated to PBS. Sections were permeabilized with Proteinase K, post-fixed in fresh 4% paraformaldehyde in PBS, and acetylated with acetic anhydride in 0.1 M triethanolamine. The tissues were then dehydrated awaiting hybridization. The hybridization conditions consisted of incubating the tissue overnight at 55°C in a 25 µl solution containing 1x Denhardt's, 10% dextran sulphate, 50% formamide, 600 µg/ml *E. coli* rRNA, 10 mM dithiothreitol, 300 mM NaCl, 10 mM Na_2HPO_4, 10 mM Tris.HCl, and 5 mM EDTA plus 1 x 10^6 cpm of [-^{35}S] UTP (Bresatec) labelled antisense RNA probes. The coverslips were removed and the tissue washed at 65°C with increasing stringency to 0.5x SSC. The tissue was dehydrated with increasing concentrations of alcohol containing 0.3 M ammonium acetate. The sections were then dipped in K5 nuclear emulsion (Ilford, UK) and exposed for 3–7 days at 4°C before development of the silver grains with developer (Phenisol, Ilford) and fixed in 30% sodium thiosulphate. Sections were counterstained with heamatoxylin and eosin. Control sections using sense-strand RNA probes and no RNA probe were also performed. Cortical distal collecting tubules were identified by the absence of a brush border membrane, a relatively large lumen and a relative abundance of cells in the tubule wall.

$1,25[OH]_2D_3$ Measurement

In order to exclude the possibility that the induction of calbindin $D28_K$ gene expression after administration of estradiol valerate in OVX animals was due to the production of $1,25(OH)_2D_3$, the effect of estradiol valerate on serum $1,25(OH)_2D_3$ levels was measured on serum taken at the time of sacrifice in separate time course studies. Serum $1,25(OH)_2D_3$ was measured using a column extraction technique followed by assay using calf thymus cytosol binding protein [Hollis, 1986]. The column extraction was carried out on a 0.5 ml–1 ml sample. An equal volume of acetonitrile was added and the precipitated protein removed by centrifugation. The supernatant was then treated with an equal volume of 0.4 M potassium phosphate, pH 10.5, and centrifuged. The supernatant from this step was added to Bond Elut C18OH columns (Varian, Harbor City, Ca) and washed with 5 ml of water, followed by methanol water (70/30 vv), and hexane dichloromethane (90/10 vv). $1,25(OH)_2D_3$ was eluted with hexane/isopropanol (95/5). The intra- and interassay coefficients of variation for the $1,25(OH)_2D_3$ assay were 14% and 20%, respectively.

RESULTS

Localization of Calbindin $D28_K$ Gene Transcript in Kidney

We performed in situ hybridisation studies using a calbindin-D28K riboprobe in OVX and

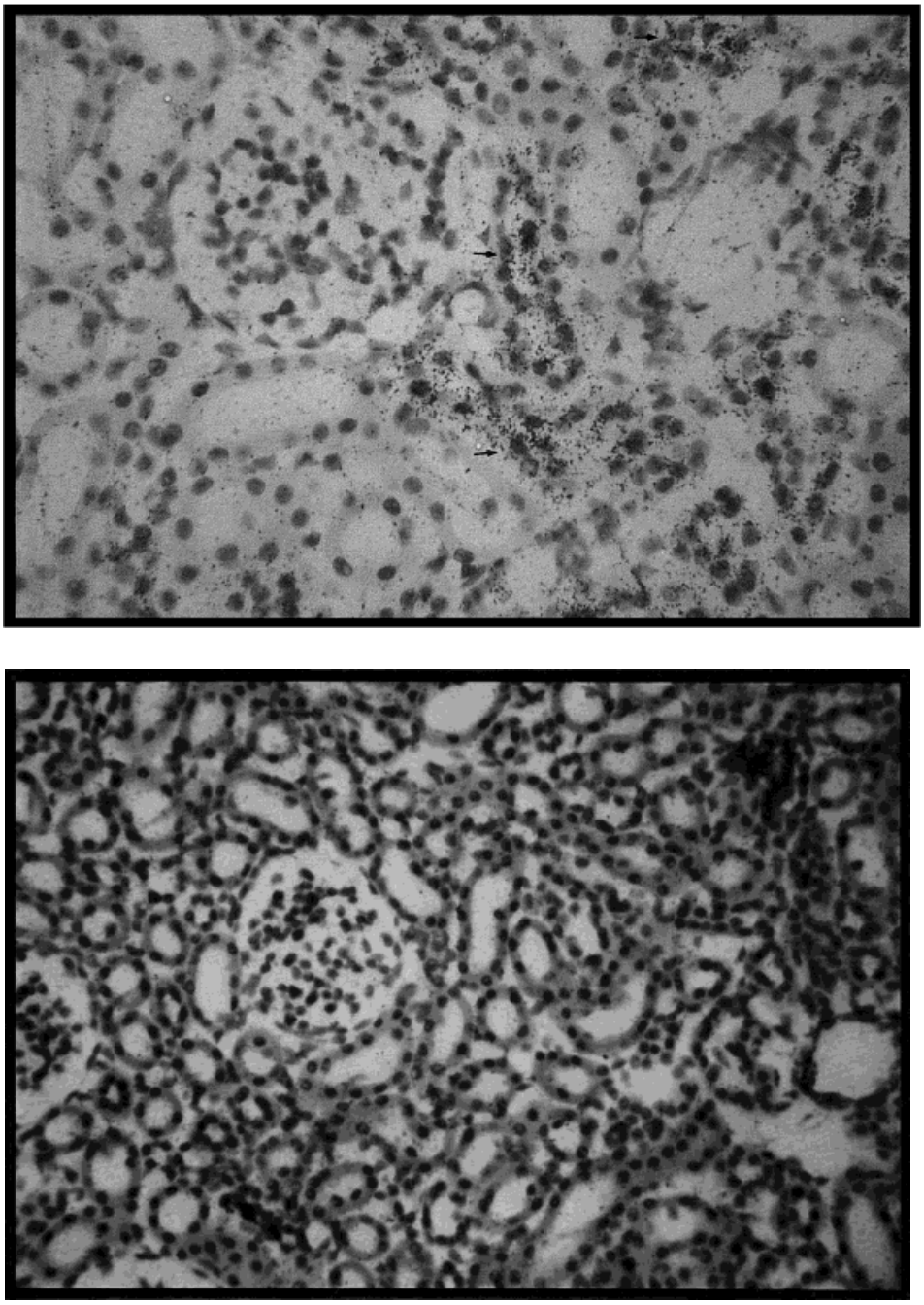


Fig. 1. Insitu hybridization of rat calbindin D_{28K} riboprobe to rat kidney in an estrogen treated OVX rat. Anitsense probe (**top**), sense probe (**bottom**). Arrows indicate the distal tubule distribution of the signal (magnification x 24).

E-OVX rats. Calbindin-D28K mRNA expression was demonstrated by the presence of silver grains as a result of hybridization with the antisense probe (Fig. 1, top). The signal was observed predominately in the cytoplasm of cortical distal tubules, confirming the distal tubular localization of calbindin $D28_{K.}$ Hybridization with sense probe labelled to the same specific activity did not result in any signal (Fig. 1, bottom). Similar results were obtained from two separate experiments.

Estrogen induced calbindin $D28_K$ gene transcripts in ovariectomized rats.

Northern blot analysis on the time course of estrogen induced renal calbindin-D_{28k} mRNA expression studied in 1 week OVX rats indicated that the level of calbindin-D_{28k} mRNA in

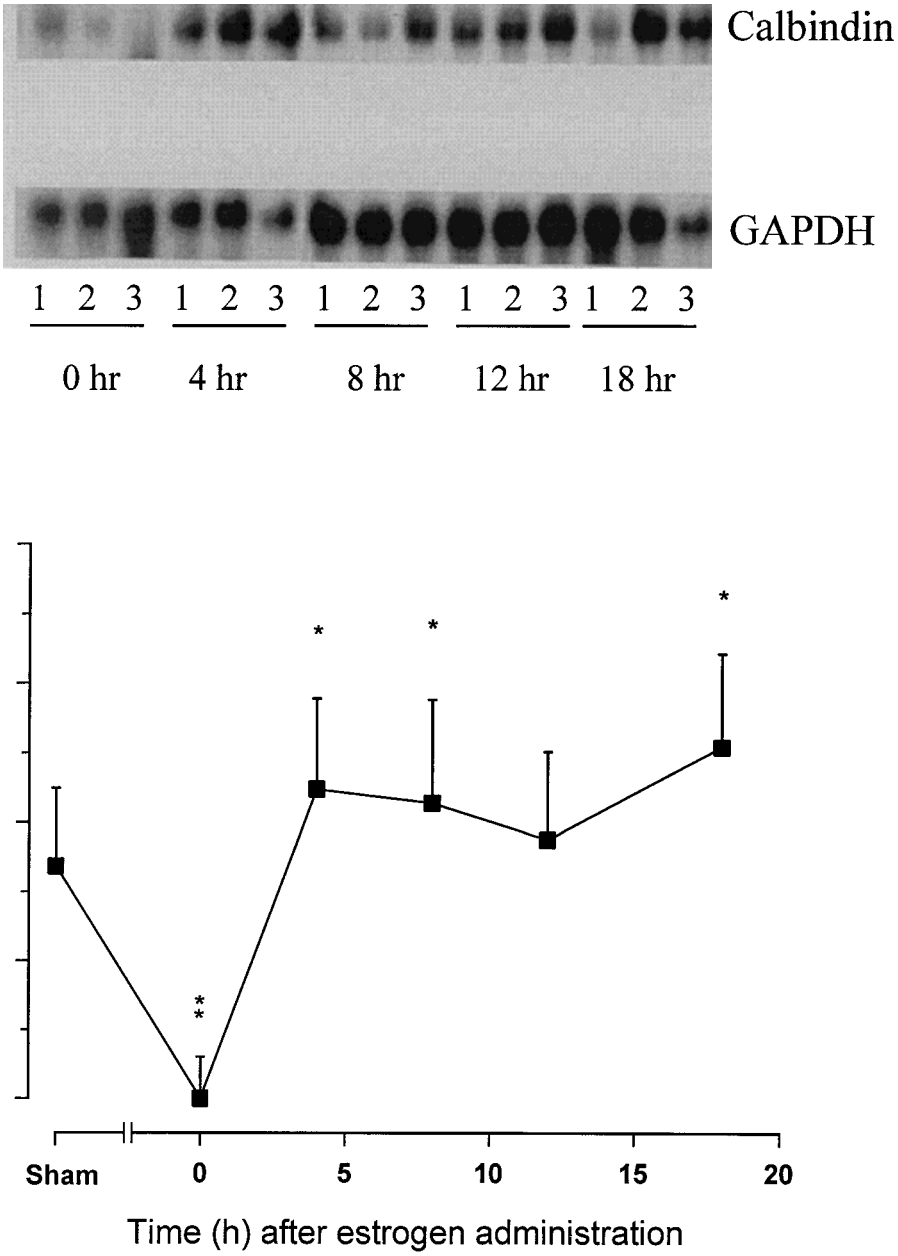
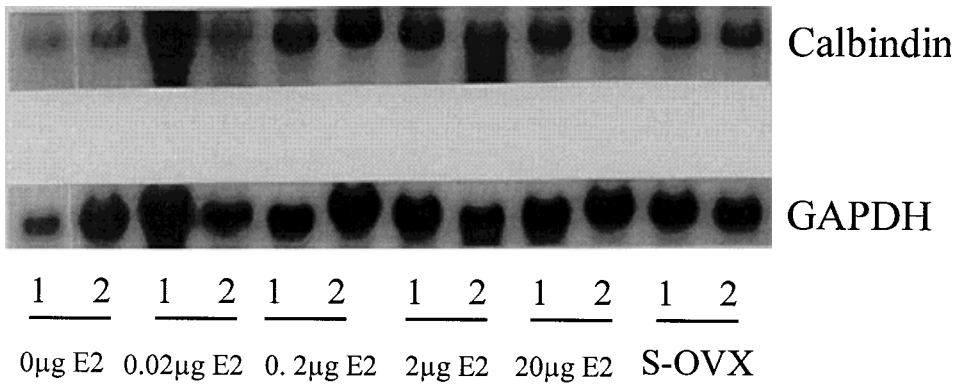


Fig. 2. Time course of the induction by estrogen valerate (20 μg) of calbindin-D28K in the OVX rat kidney. Results are mean ± SEM of the fold increase in the maximal densitometric response (n = 5–9) of the calbindin-D28K/GAPDH ratios. *$P < 0.05$ one way ANOVA—Duncan's multirange test comparing time points with 0 h. **$P < 0.03$ unpaired *t*-test comparing sham operated rat with 0 h. The upper panel shows a representative northern blot with calbindin D_{28K} above GAPDH on the same membrane.

sham operated animals was higher than in the OVX nonestrogen treated ($P < 0.03$ *t*-test) (Fig. 2). There was a significant rise in calbindin-D28k expression by 4 h which was sustained at all subsequent times measured (Fig. 2). The dose response study indicated that at all doses measured greater than 0.2 μg, estradiol valerate appeared to increase calbindin-D28K mRNA expression (Fig. 3). It is noteworthy that there was an apparent difference in the relative levels of calbindin-D_{28k} mRNA compared to calbindin-D_{28k} mRNA from E-OVX and OVX rats between the time course and dose response experiments.

Evidence that induction of calbindin $D28_K$ gene expression by estrogen is not mediated by $1,25(OH)_2D_3$ production.

As renal calbindin-D28K mRNA expression is known to be stimulated by $1,25(OH)_2D_3$, we



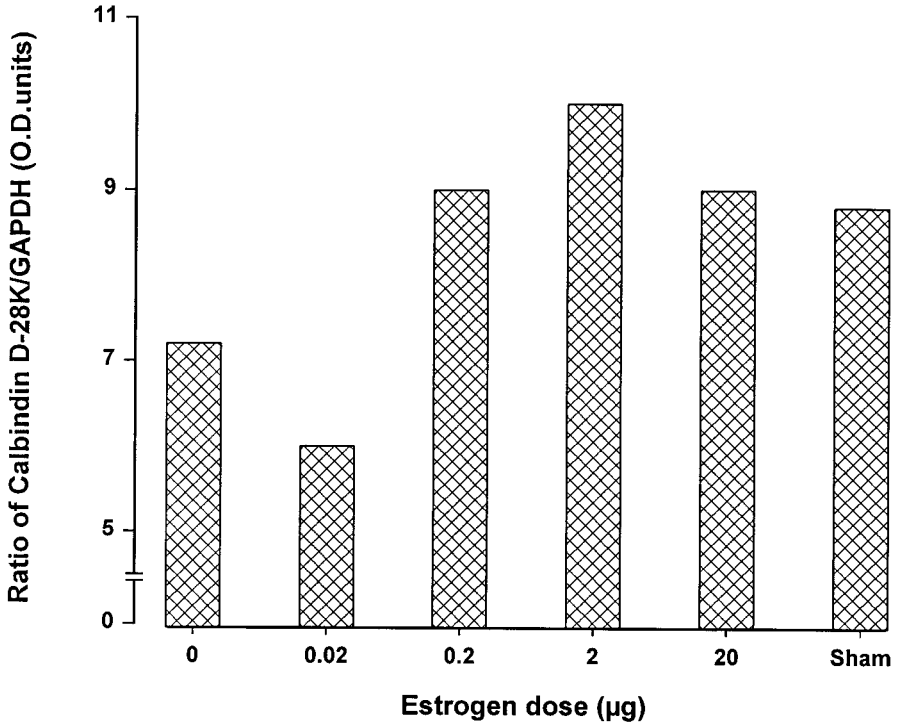


Fig. 3. Dose response of the induction by estrogen of calbindin-D28K in the OVX rat kidney. Results are the mean (n = 2) of the calbindin-D28K/GAPDH densitometric response. The upper panel shows a representative northern blot.

measured 1,25-$(OH)_2D_3$ levels at 2, 4, 8, 12, and 18 h after injection with 20 μg of estradiol valerate (Fig. 4). 1,25$(OH)_2D_3$ levels were significantly higher is the sham operated animals but no change was seen after estrogen injection of ovariectomized rats.

DISCUSSION

The kidney has an important role in calcium balance in humans as it filters more than 8,000 mg of calcium each day and reabsorbs almost 98% of that load [Heaney, 1990]. Small changes in the percentage reabsorption of calcium if sustained over a long period may have a dramatic effect on total body calcium stores. Thiazide diuretics, which reduce renal calcium excretion, have been shown to increase bone mineral density [Wasnich et al., 1983; Felson et al., 1991], an observation supporting a link between renal calcium conservation and bone strength.

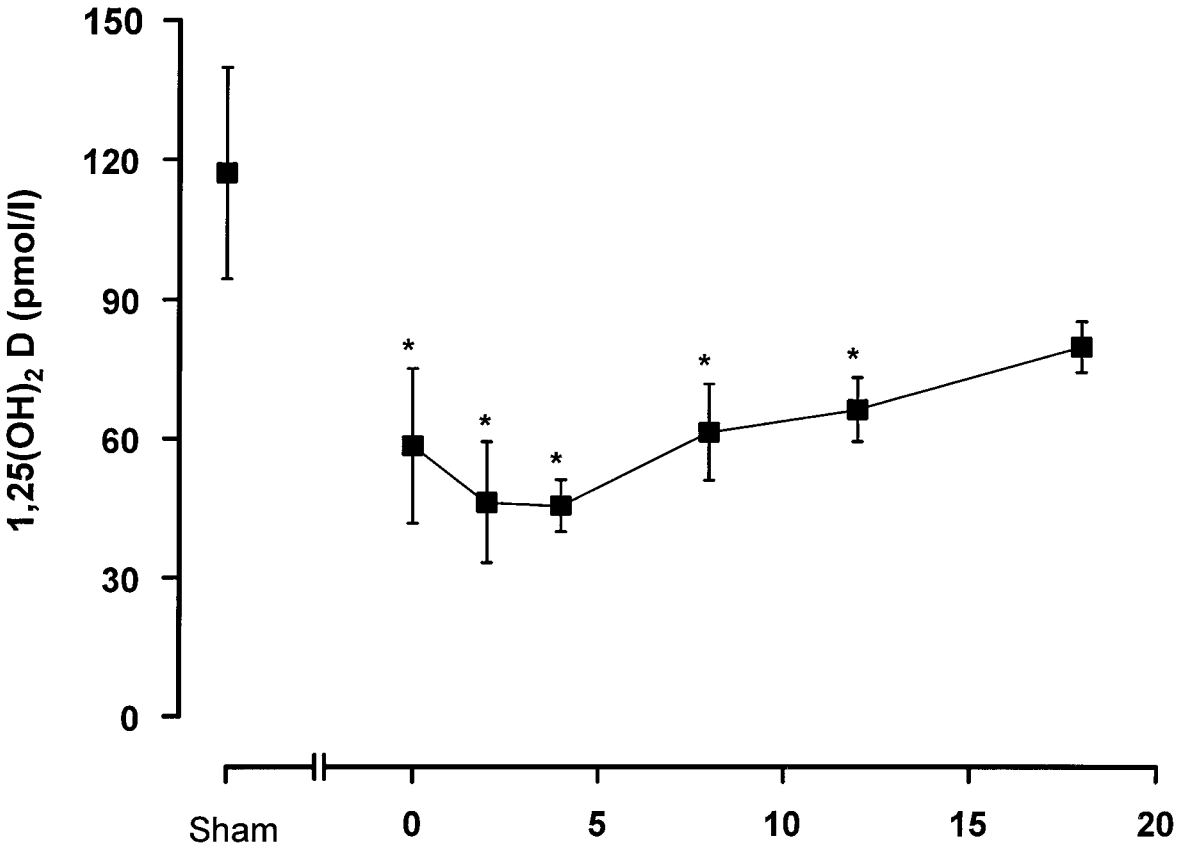


Fig. 4. Time course of $1,25(OH)_2D_3$ response in the OVX rat to a 20 μg dose of estrogen. Animals were sacrificed at 0, 2, 4, 8, 12, and 18 h after injection. Results are mean ± SEM (n = 3). *$P < 0.05$ by one way ANOVA—Duncan's multirange test compared to sham group.

Previous studies in the human have suggested that the estrogen deficiency occurring at the menopause is associated with a renal calcium loss that is due to a direct effect of estrogen on the kidney to increase renal calcium loss [Nordin et al., 1991; Adami et al., 1992]. This suggests that estrogen deficiency results in an increased renal calcium loss in the human and it has been shown that this "renal calcium leak" is corrected with estrogen replacement [Prince et al., 1991; Adami et al., 1992; McKane et al., 1995].

The results of the present study demonstrate for the first time that ovariectomy in the rat leads to a reduction in mRNA levels of renal calbindin D_{28K}. The mRNA level in the OVX rat was approximately three times lower than the S-OVX rats. Our results also indicate that administration of 17β estradiol was effective in increasing mRNA levels within 4 h. It is important to exclude a possible effect of $1,25(OH)_2D_3$ on calbindin D_{28K} mRNA expression in view of evidence showing an effect of oral estrogen to increase $1,25(OH)_2D_3$ levels in post menopausal women [Cheema et al., 1989]. This rise in $1,25(OH)_2D_3$ is largely a consequence of an increase in vitamin D binding protein that results from oral estrogen administration, an effect that is not observed with transdermal estrogen administration [Dick et al., 1995]. The finding that serum $1,25(OH)_2D_3$ levels, which were lower in OVX rats than S-OVX rats, did not rise with 17β estradiol administration indicate that our findings were not mediated by $1,25(OH)_2D_3$. This is not surprising, as previous evidence indicates that estrogen stimulates $1,25(OH)_2D_3$ production by effects on calcium balance [Prince, 1994] and not as a result of a direct effect on $1,25(OH)_2D_3$ production [Henry, 1981]. The in situ hybridisation studies indicate that the calbindin D_{28K} mRNA expression being detected resides within the distal tubular cell as previously described [Rhoten and Christakos, 1990]. Furthermore, there is evidence that classical estrogen receptors reside in the kidney [Hagenfeldt and Eriksson, 1988] and that an estrogen

response element exists upstream of the mouse calbindin D_{28K} gene [Gill and Christakos, 1995]. These results are therefore in keeping with a direct effect of estrogen on the distal tubular cells of the kidney. Clearly the increased calbindin D_{28K} mRNA levels in E-OVX rats could be due to increased transcription of calbindin D_{28K} or stabilisation of mRNA with reduced degradation. In view of previous evidence that alterations in renal calbindin D_{28K} mRNA levels by $1,25(OH)_2D_3$ are associated with increased calbindin D_{28K} protein levels [Chen et al., 1992], it is highly likely that estrogen also modulates calbindin D_{28K} protein levels.

A resulting increase in calbindin D_{28K} as a result of the action of estrogen could be associated with increased trancellular calcium transport by a number of potential mechanisms. Calbindins may act to bind intracellular calcium, so as to maintain the ionized calcium gradient between the apical and basolateral membranes. It is postulated that it then releases its bound calcium at the basolateral membrane to the plasma membrane calcium ATPase pump, which has a higher affinity for calcium, for extrusion from the cell [Johnson and Kumar, 1994]. In support of this concept, it has been demonstrated using flow-through dialysis that calbindin $D9_K$ increases calcium flux [Feher, 1983]. Calbindin D_{28K} has been shown to enhance the transfer of calcium across the apical membrane of both the distal and proximal tubule [Bouhtiauy et al., 1994], but as calbindin D_{28K} is only found in the distal tubule, the proximal effect is unlikely to be of physiological significance. Calbindin D_{28K} also increases the activity of the purified plasma membrane enzyme calcium ATPase [Reisner et al., 1992] although the physiological importance of this has not been demonstrated [Bouhtiauy et al., 1994].

Previous studies of estrogen receptor localisation have suggested the glomerulus, interstitial cells, and vascular epithelium in the guinea pig [Bhat et al., 1993] or the proximal tubule in the rat [Stumpf et al., 1980] as possible sites. Our findings with regard to renal calbindin D_{28K} mRNA expression provide further evidence of estrogen responsiveness in rat kidneys. This study shows changes in the mRNA of a functionally important protein in the distal renal tubule in response to estrogen. This supports our hypothesis that the kidney is an estrogen responsive organ that may be a site of important changes in calcium metabolism following the menopause. Further studies in cell culture of distal tubular cells will help to delineate the mechanism of the changes we have identified in the intact animal model.

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