Journal of Cellular Biochemistry 70:84–93 (1998)

Estrogen Stimulates PTHrP But Not PTH/PTHrP Receptor Gene Expression in the Kidney of Ovariectomized Rat

Magali Cros,[1]* Caroline Silve,[2] Anne-Marie Graulet,[3] Caroline Morieux,[1] Pablo Ureña,[4] Marie-Christine de Vernejoul,[1] and Zhor Bouizar[1]

[1]INSERM U349, Hôpital Lariboisière, Paris, France
[2]INSERM U426, Hôpital Xavier Bichat, Paris, France
[3]Laboratoire de Radioimmunologie, Hôpital Lariboisière, Paris, France
[4]Clinique de l'Orangerie, Aubervilliers, France

Abstract The aim of the present study was to test the hypothesis that the decreased renal tubular reabsorption of calcium observed in estrogen deficiency is associated with a local regulation of either PTHrP or PTH/PTHrP receptor genes in the kidney. Rats were randomly sham-operated (S) or ovariectomized receiving either vehicule (OVX) or 4 µg E2/kg/day (OVX+E4) or 40 µg E2/kg/d (OVX+E40) during 14 days using alzet minipumps. Plasma PTH and calcium levels were lower in untreated OVX animals than in all other groups ($P < 0.01$). Plasma PTH was higher in OVX+E40 than in OVX+E4 ($P < 0.05$). PTHrP mRNA expression in the kidney was unaffected by ovariectomy but was increased in OVX+E40 (0.984 ± 0.452 for PTHrP/GAPDH mRNAs expression vs. 0.213 ± 0.078 in sham, $P < 0.01$). PTH/PTHrP receptor mRNA expression and the cAMP response of renal membranes to PTH were unaffected by ovariectomy and estrogen substitution. In conclusion, renal PTHrP and PTH/PTHrP receptor mRNAs are not modified by ovariectomy. However, 17β-estradiol increases renal expression of PTHrP mRNA without evident changes in its receptor expression and function. This may help to explain the pharmacological action of estrogen in the kidney, especially how it prevents the renal leak of calcium in postmenopausal women. J. Cell. Biochem. 70:84–93, 1998.

Key words: PTHrP; PTH/PTHrP receptor; estrogen; ovariectomy; kidney; rat; in vivo

In women, menopause results in an estrogen deficiency. The major consequence is a bone loss associated with a renal leak in calcium [Nordin et al., 1991] and subsequent consecutive osteoporosis. Estrogen substitutive therapy limits this decrease in bone mass. In addition to this role on the skeleton, it has been suggested that estrogen may act on calcium homeostasis, regulating renal calcium reabsorption [Adami et al., 1992; Prince, 1994], but the mechanisms are still poorly understood.

Parathyroid hormone–related peptide (PTHrP) is generally responsible for humoral hypercalcemia of malignancy [Suva et al., 1987]. While many tumor types overexpress PTHrP, it is also produced by a variety of normal tissues, including the central nervous system, epidermal keratinocytes, lactating breast, placenta, and vascular smooth muscle [Kramer et al., 1991]. PTHrP is also expressed in fetal mice [Lee et al., 1995], where it seems to play an important role in normal growth and development, as shown by the lethal skeletal dysplasia resulting from its gene disruption [Karaplis et al., 1994; Amizuka et al., 1994]. In addition, PTHrP mRNA and proteins have been identified in specific zones of the fetal and adult kidney. In adult kidney, PTHrP has been histochemically located in glomerular epithelial cells and in proximal, distal, and collecting tubule cells [Garcia-Ocaña et al., 1995]. The rat PTHrP gene has been characterized [Karaplis et al., 1990]. This gene consists of only four exons. The first exon encodes an alternative 5' untranslated region containing several GC-rich sequences, a TATA box and a CAAT box, and several Sp1 binding sites. The second exon encodes the prepro sequence. The third exon encodes a 139 amino

*Correspondence to: Magali Cros, Laboratoire de Biologie Cellulaire et Moléculaire de l'Os et du Cartilage, INSERM U349, Hôpital Lariboisière, 6 rue Guy Patin, 75475 Paris Cedex 10, France.

Received 21 November 1997; Accepted 9 February 1998

acid sequence of the mature peptide, and the fourth exon encodes for two extra amino acids, the stop codon and the 3' untranslated region. It is simpler than in human but shows a strong conservation of nucleotide sequence and amino acid sequence homology up to residue 111.

Parathyroid hormone (PTH) (1-34) and PTHrP(1-34) share similarities at the amino acid sequence level as well as in three-dimensional homology, accounting for the fact that these two peptides act through the same PTH/PTHrP receptor [Suva et al., 1987]. The rat PTH/PTHrP receptor has been cloned [Abou-Samra et al., 1992]; it is a member of the seven transmembrane domain receptors coupled to a G protein [Jüppner et al., 1991] and activates at least two signal transduction pathways: the adenylyl cyclase and the protein kinase C pathways. This receptor is distributed as widely as PTHrP [Ureña et el., 1993], pointing to an autocrine-paracrine role for PTHrP in normal physiology. Indeed, it has been demonstrated that PTHrP regulates cell growth and differentiation (in vitro and in vivo), relaxation of smooth muscle cells, and vasodilation [Garcia-Ocaña et al., 1995; Soifer et al., 1993; Nyby et al., 1995; Shew et al., 1991]. Little is known about the renal regulation of PTHrP expression, and its precise role in the kidney is not well defined. In the kidney, it has been shown that PTHrP, acting through the PTH/PTHrP receptor, can mimic PTH actions, in particular by stimulating calcium reabsorption by renal tubular cells [Yates et al., 1988]. PTHrP is overexpressed in the kidney after renal ischemia [Soifer et al., 1993] and accelerates the growth of cultured rabbit proximal tubule cells [Garcia-Ocaña et al., 1995]. These last observations support the hypothesis that PTHrP secreted by tubule cells plays an important role in the regulation of tubular cell growth during regeneration after ischemia.

A recent study suggested that estrogen treatment may play a role in the increase of renal tubular reabsorption of calcium which results in a decrease in urinary calcium excretion [Criddle et al., 1997]. As it is known that estrogen increases PTHrP mRNA expression in vivo in the rat uterus and given to the role played by the PTH/PTHrP receptor in renal calcium handling, we hypothesized that the reduction of the renal leak of calcium oberved during estrogen substitutive treatment [Adami et al., 1992] may be associated with a local change in PTHrP and/or PTH/PTHrP receptor expression.

To test this hypothesis, we used the model of the ovariectomized mature female rat for estrogen deficiency, and we measured the renal expression of PTHrP and PTH/PTHrP receptor genes expression in sham-operated, ovariectomized untreated, and ovariectomized estrogen-treated rats using a physiological and a pharmacological dose of 17β-estradiol.

MATERIALS AND METHODS

Animals and Treatment

The experiment was performed twice. We once randomized 18 and once 40 3-month-old Sprague-Dawley (S.D.) female rats (Charles River Laboratories, St. Aubin les Elbeuf, France), respectively, into three groups of six animals (there was no OVX+E4 group) and into four groups of ten animals. Animals were kept under 12 h light-dark cycles. After anesthesia with an intraperitoneal injection of Imalgene (Rhône-Biomérieux, Lyon, France), a sham operation or ovariectomy was performed, and a dorsal subcutaneous minipump (Alzet, France) was inserted into OVX animals to deliver 17β-estradiol (E2) (Sigma, St. Quentin-Fallavier, France) or 50% DMSO/0.045% NaCl (vehicle) alone. Sham-operated animals (S) served as controls. Ovariectomized animals were given either the vehicule alone (OVX), or 4 µg E2/kg of body weight/day (OVX+E4) (only for the second experiment) or 40 µg E2/kg/day (OVX+E40). Treatment was started immediately after ovariectomy and lasted 14 days.

Tissue Preparation

Kidneys from anesthetized rats were either frozen in liquid nitrogen for total RNA extraction or cytosol extraction or fixed in 4% paraformaldehyde (PFA) for in situ hybridization. Fixed kidneys were rinsed in 5% sucrose in phosphate buffered saline (PBS) and transferred to 30% sucrose in PBS for at least 3 h before embedding in Tissue-Teck 2 (Miles Laboratories, Epernon, France) and freezing in liquid nitrogen. Five micrometer sections of fresh-frozen kidneys were cut on a cryotome (Frigocut 2700; Microm, Venissieux, France) and collected on Vectabond-coated (Superfrost; CML, Nemours, France) glass slides.

Biochemical Analyses

Serum total calcium and serum creatinine were determined using a standard method on a Kodak analyzer. Serum PTH was measured with a rat PTH (1-34) radioimmunoassay kit (Peninsula Laboratories, Belmont, CA). 17β-estradiol was assayed with a radioimmunoassay kit (Sorin Biomedica, Antony, France). PTHrP was evaluated in cytosol extracts of kidneys using a human-PTHrP (1-34) immunoradiometric assay (Peninsula Laboratories).

Preparation of Total RNA

Total RNA was extracted by using a modification of a previously described method [Chomiczynski and Sacchi, 1987]. Kidneys were homogenized in a solution containing isothiocyanate guanidium and phenol (RNA Now; Biogentex, Montigny-le-Bretonneux, France) with a Polytron grinder (Scientific Bioblock, Illkirch, France). Chloroform was added, and the aqueous phase was separated by centrifugation for 10 minutes at 12,500*g*. Total RNA was precipitated by adding isopropanol to the aqueous upper phase and centrifuging at 12,000*g* for 15 min. The pellet was washed twice with 70% ethanol, dried in a Speed-vac, and resuspended in RNAse-free water (treated with diethyl pyrocarbonate and sterilized). Total RNA was determined by measuring absorbance at 260 nm.

Ribonuclease Protection Assay

The ribonuclease protection assay (RPA) was performed essentially as described elsewhere [Suarez et al., 1995]. The plasmid containing the rat PTHrP fragment (kindly provided by Dr. Thiede) was linearized by digestion with *Hind*III (Gibco-BRL, Cergy-Pontoise, France). To prepare the antisense riboprobe, we transcribed 1 µg of linearized plasmid in the presence of 50 µCi [α-^{32}P]UTP (400 Ci/mmol) (Amersham, Les Ules, France) using T7 RNA polymerase and reagents from the Gemini System (Promega, Charbonniéres, France) in a final volume of 20 µl. An antisense rat GAPDH probe was prepared by using a plasmid containing the specific rat GAPDH cDNA fragment digested with *Sty*I and *Pvu*II (Promega, France). For the analysis of PTHrP and GAPDH mRNAs, 100 µg of total RNA was hybridized overnight at 55°C with 5×10^5 cpm of rat PTHrP and 5×10^4 cpm of rat GAPDH riboprobes (in a final volume of 30 µl) and digested in 350 µl of RNase digestion buffer containing 40 µg/ml RNase A and 2 µg/ml RNase T1 (Boehringer-Mannheim, Meylan, France). RNA fragments were electrophoresed in 6% polyacrylamide-urea gels in parallel with an [α-^{32}P]UTP-labeled RNA molecular weight marker (RNA Century Marker Plus template set; Ambion, CliniSciences, Montrouge, France). Gels were dried, and autoradiograms were prepared by exposure to films (NEF-485; NEN, NEN Life Sciences, Le Blanc-Mesnil, France) for 7 days at -80°C with two intensifying screens. The unprotected PTHrP riboprobe migrated at 400 bp and the protected fragment at 330 bp. The GAPDH riboprobe migrated at 192 bp when unprotected and at 164 bp when protected.

The protected mRNA fragments were quantified by counting radioactivity directly on dried gels using an electronic autoradiographer (in Instant Imager; Packard, France). Bands obtained with PTHrP and GAPDH riboprobes were quantified Rungis. Background was evaluated to obtain the specific radioactivity of each signal. Results were expressed in arbitrary units as the ratio of PTHrP to GAPDH mRNA-specific signals. In each experiment riboprobes were incubated with yeast transfer RNA (Boehringer-Mannheim) before RNase digestion, and fragments were undetectable in these samples. RNAse protection was realized twice but only in the experiment with 40 animals.

Reverse Transcription and Polymerase Chain Reaction Analysis

Four micrograms of total RNA were submitted to reverse transcription by incubation for 10 min at 23°C and 1 h at 37°C in a final volume of 20 µl containing 1 mM each dNTP (dNTP set; Pharmacia, Orsay, France), 18 U of RNAse inhibitor (RNAsin; Promega), 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL), 50 pmoles of oligo-dT primer (Promega), 0.01 M DTT (Gibco BRL), and 4 µl of 5× first-strand buffer (Gibco BRL). Incubation was done in a programmable thermal cycler (Techne, France).

Nine microliters of cDNA was submitted to amplification of rat PTHrP or rat PTH/PTHrP receptor using 4 µl of 10× PCR incubation buffer (Appligene, Illkirch, France) and 0.5 U of *Thermophilus aquaticus* polymerase (Appligene). We used 25 pmol of sense (5′ TCTTCCTCCACCATCTGATTGC 3′) and antisense (5′ CAGAGTCAGCAGCACCAAGATAC 3′) prim-

ers (Genosys, Combs, UK) to amplify a 611 bp fragment of the rat PTHrP cDNA. Sense (5′ GTGTCAGAGCAACCTTGG 3′) and antisense (5′ AACCGCCATGACTGG 3′) primers were used to amplify a 641 bp fragment of the rat PTH/PTHrP receptor cDNA. All these oligonucleotides were located in two different exons. Amplification was performed for 25 cycles (PTHrP cDNA) or 30 cycles (PTH/PTHrP receptor cDNA) based on a kinetic study of the amplification reaction. The reaction started with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. The rat GAPDH cDNA (ubiquitous internal control) was amplified using sense (5′ ATTGTTGCCATCAACGACC 3′) and antisense (5′ CATGGACTGTGGTCATGAGC 3′) oligonucleotide primers to obtain a 450 bp fragment. Amplification was performed as described for PTHrP.

Southern Blot and Hybridization

Amplification products were electrophoresed on 2% agarose gel in Tris-EDTA-Borate buffer, pH 8. A DNA molecular weight marker was used to estimate band sizes (Marker VI; Boerhinger-Mannheim). DNA fragments were visualized by ethidium bromide staining under UV light. DNA fragments were transferred to a nylon membrane (GeneScreen; NEN) using 0.4 N NaOH as described in the protocol recommended by NEN.

Rat PTHrP, PTH/PTHrP receptor, and GAPDH cDNA probes were [α-^{32}P]-labeled using a random labeling system (Prime-a-gene; Promega) with [α-^{32}P]-dCTP (3,000Ci/mmol) (NEN). Each membrane was incubated with 6×10^6 cpm of the corresponding specific probe in hybridization solution (6× SSC, 50 µg/ml yeast tRNA, 2× Denhardt's solution, 10% sonicated herring sperm DNA) overnight at 42°C. Nonspecific radioactivity was eliminated by washing in 0.2× SSC/1% SDS at 42°C. Membranes were then autoradiographed.

The films (NEF-485; NEN) were analyzed on a densitometric scanner (Transidyne General Corporation, Roucaire, Velizy-Villacoublay, France), and results were expressed in arbitrary units as the ratio of PTHrP or PTH/PTHrP receptor to GAPDH densitometric values. RT-PCR, Southern blot, and hybridization of the membranes were realized at least two times in each experiment.

Riboprobe Labeling and In Situ Hybridization

Sense and antisense rat PTH/PTHrP receptor riboprobes were synthesized and ^{35}S-UTP–labeled with a riboprobe labeling system (Gemini II Core System; Promega) and α-^{35}S-UTP (10 mCi/ml) (NEN) from clone R15B (kindly provided by G.V. Segre) after linearization with *Xba*I (Gibco-BRL) for the sense strand or BamHI (Promega) for the antisense strand and using T7 and SP6 RNA polymerases, respectively. Cryosections were postfixed with freshly prepared 4% PFA/0.01 M PBS for 15 min. After washing in PBS, fixed sections were digested with 2 µg/ml proteinase K at 37°C for 15 min and again treated with 4% PFA/PBS for 10 min. Sections were sequentially washed with PBS, incubated with 0.2 N HCl for 10 min, washed with PBS, acetylated with 0.3% acetic anhydride in the presence of 0.1 M triethanolamine for 10 min, gradually dried in ethanol, and air-dried.

Hybridization with 10^6 cpm/section ^{35}S-labeled riboprobe was performed in hybridization solution containing 50% formamide, 10% Dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 10 mM Tris-HCl, 0.25% SDS, 1 mM EDTA, pH 8.0, and 200 µg/ml tRNA in a 50% formamide/50% H_2O atmosphere for 17 h at 55°C.

After hybridization, sections were washed, treated with 10 µg/ml RNase A (37°C for 30 min), and dehydrated in ethanol. Slides were exposed to films (Hyperfilms β-max; Amersham) for 48 h, and autoradiograms were quantified on an image analyzer (Biocom, Les Ulis, France). The slides were then dipped in autoradiographic emulsion (NTB-2, Integra-Biosciences, Eaubonne, France) for 4 days. After development, sections were stained with toluidine blue and mounted with Fluoromount for X-ray observation. In situ hybridization was repeated at least two times but only in the experiment with 18 animals.

Preparation of Renal Cortical Membranes for Adenylate Cyclase Stimulation and Cyclic AMP Assay

Cortical membranes were extracted as previously described [Marx et al., 1972]: the renal cortex was dissected free and homogenized in buffer containing 0.25 M sucrose, 10 mM Tris, and 1 mM Na_2EDTA, pH 7.5. The homogenized tissue was centrifuged as follows: the rotor was

stopped when it reached 2,500*g*, and the supernatant was collected and centrifuged at 2,500*g* for 20 min. The pellet was suspended in buffer containing 10 mM Tris and 1 mM Na_2EDTA, pH 7.5. Protein content was measured using the Bio-Rad kit (Bio-Rad, Paris, France) based on Bradford's method.

Renal membrane preparations containing 75 µg of protein were added to 50 mM Tris, pH 7.5, 1.7 mM $MgCl_2$, 100 mM 3-isobutyl-1-methylxanthine (IBMX), 0.1% BSA, 7 mM phosphocreatine, 1.2 mM ATP, and 1 U creatine kinase in a final volume of 100 µl. This mixture was incubated for 30 min at 37°C with either 1 mM acetic acid, 1 µM (1-34)bPTH (Sigma), 30 µM forskolin (Sigma), or 10 µM isoproterenol (Sigma). The reaction was stopped by heating at 95°C for 3 min, and protein was removed by adding 400 µl of absolute ethanol. Tubes were centrifuged for 5 min at 10,000*g*, and the supernatant was collected and evaporated overnight at 37°C. The resulting pellet was dissolved in 500 µl of 50 mM sodium acetate, pH 6.2.

Cyclic AMP accumulation was determined by radioimmunoassay (cAMP[^{125}I] RIA kit; NEN) after acetylation procedure. Adenylate cyclase stimulation and cAMP assay were repeated at least two times but only in the experiment with 18 animals.

Statistical Analysis

As identical significative differences were observed between groups for the two experiments, results were pooled together and expressed as the mean ± SEM of both experiments. The data were analysed by one-way ANOVA for multiple comparison. The nonparametric Mann-Whitney U-test was used when values were not assessed to be Gaussian. $P < 0.05$ was considered significant.

RESULTS

Animals and Biochemicals Parameters

After sacrifice, untreated OVX animals weighed significantly more (311 ± 6.9 g) than those in the other groups (273 ± 2.8 g for sham, 283 ± 7.1 g for OVX+E4). Treatment with 40 µg E2/kg/day (OVX+E40) induced a significant reduction in weight (258 ± 6.3 g) ($P < 0.05$).

Biochemical results are summarized in Table I. OVX induced a slight but significant reduction in plasma PTH (28.3 ± 0.8 pg/ml vs. 31.9 ± 1.1, $P < 0.05$) and total calcium (2.46 ± 0.03 mmol/l vs. 2.59 ± 0.06, $P < 0.01$) compared with the sham group. Total calcium returned to sham-control levels in OVX+E4 and OVX+E40 groups (2.65 ± 0.05 mmol/l and 2.64 ± 0.04 mmol/l). Plasma PTH returned to sham levels in estrogen-treated animals but was significantly increased with 40 µg E2/kg/day (32.6 ± 1.1 pg/ml, $P < 0.05$) compared to untreated OVX.

Levels of 17β-estradiol were significantly lower in OVX (5.2 ± 0.8 pg/ml) and significantly higher in OVX+E40 (58.0 ± 9.2) than in the sham (17.9 ± 2.5), and OVX+E4 (15.5 ± 1.3) groups ($P < 0.01$).

PTHrP Gene Expression Regulation by Ovariectomy and Estrogen

PTHrP mRNA levels were unmodified in OVX (103 ± 12% vs. sham) and OVX+E4 animals (85 ± 6% vs. sham) groups but were markedly increased in OVX+E40 (288 ± 28% vs. sham, $P < 0.0001$) when compared to the levels measured in sham controls as shown by RT-PCR analysis (Fig. 1).

TABLE I. Biochemical Parameters Obtained at Sacrifice[a]

	S	OVX	OVX + E4	OVX + E40
PTH (pg/ml)	31.9 ± 1.1 (15)	28.3 ± 0.8* (15)	30.6 ± 0.8 (10)	32.6 ± 1.1 (16)
Calcium (mmol/l)	2.59 ± 0.06 (11)	2.46 ± 0.03** (15)	2.65 ± 0.05 (9)	2.64 ± 0.04 (15)
E2 (pg/ml)	17.9 ± 2.5 (15)	5.2 ± 0.8*** (15)	15.5 ± 1.3 (10)	58.1 ± 9.2*** (16)
Creatinin (µmol/l)	46.3 ± 2.7 (15)	43.0 ± 3.3 (15)	51.5 ± 2.4 (10)	48.3 ± 3.9 (16)

[a]PTH, immunoreactive rat (1—34)–parathyroid hormone; E2, 17β-estradiol; S, sham-operated; OVX, ovariectomized; OVX + E4, ovariectomized receiving 4 µg E2/kg/day; OVX + E40, ovariectomized receiving 40 µg E2/kg/day. The number of animals is in parentheses.

*$P < 0.01$ compared to sham and OVX + E40.

**$P < 0.01$ compared to all other groups.

***$P < 0.001$ compared to all other groups.

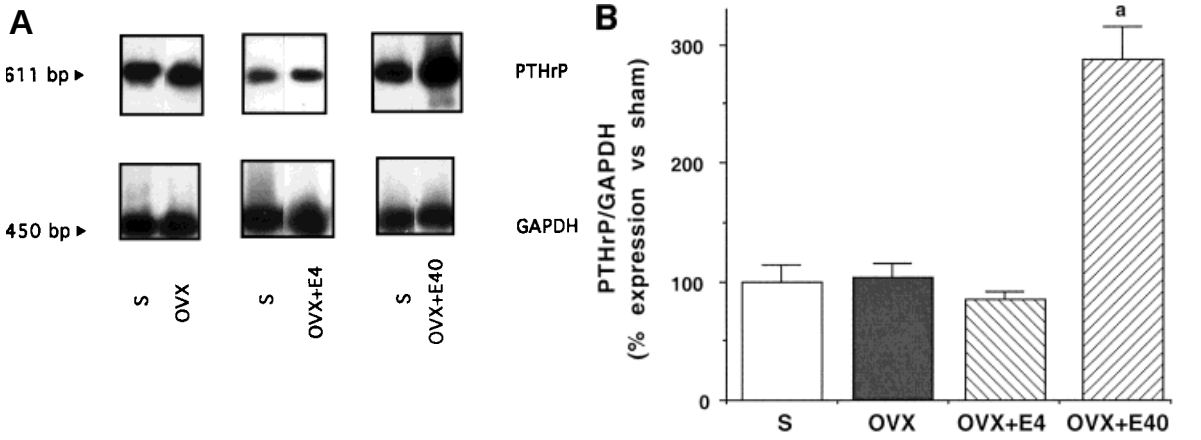

Fig. 1. Semiquantitative RT-PCR analysis of PTHrP mRNA expression in the kidney. bp, base pairs; OVX, ovariectomized (n = 15); OVX+E4, ovariectomized receiving 4 µg E2/kg/day (n = 10); OVX+E40, ovariectomized receiving 40 µg E2/kg/day (n = 13); S, sham-operated (n = 15). **A:** Southern blot analysis of PCR products was realized as decribed in Materials and Methods. One representative sample is shown for each group. Primers were used to amplify a 611 bp PTHrP fragment and a 450 bp GAPDH fragment. **B.** Quantitative results are presented as the ratio of PTHrP to GAPDH (internal standard). Results shown are mean ± SEM of the percentage of expression from sham controls. [a]$P < 0.0001$ compared with sham.

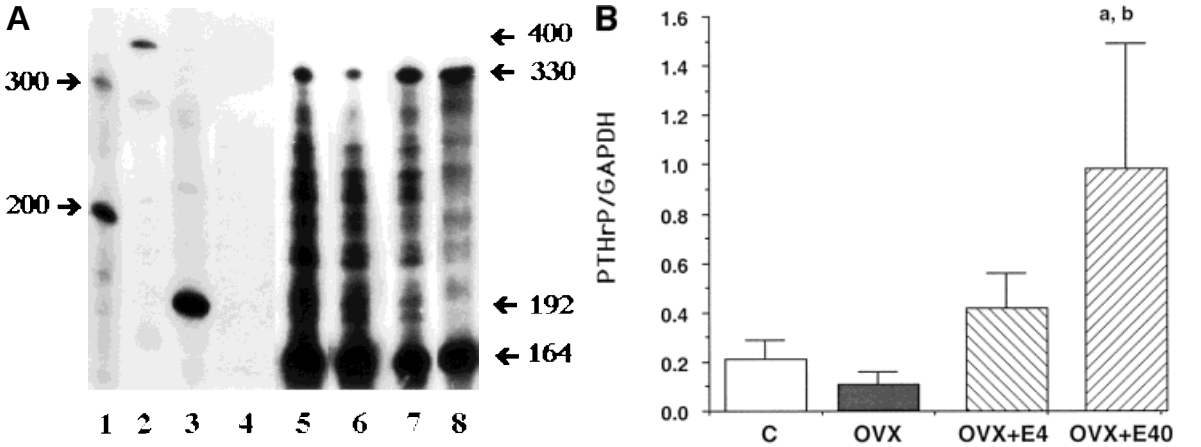

Fig. 2. PTHrP mRNA expression analysis by ribonuclease protection assay. **A:** RNase protection analysis gel realized as described in Materials and Methods. One representative migration of the molecular weight marker (**lane 1**), the 400 bp PTHrP (**lane 2**) and the 192 bp GAPDH (**lane 3**) unprotected riboprobes, a specific hybridization with PTHrP and GAPDH riboprobes of the negative control yeast tRNA (**lane 4**), and a representative hybridization sample of the sham-control (**lane 5**) (n = 7), OVX (**lane 6**) (n = 6), OVX+E4 (**lane 7**) (n = 7), and the OVX+E40 (**lane 8**) (n = 5) groups. Digested hybridized products are a 330 bp PTHrP fragment and a 164 bp GAPDH fragment. **B:** Quantitative results are evaluated in cpm using electronic autoradiography and are presented as the ratio of PTHrP to GAPDH (internal standard). Results shown are mean ± SEM of each group. [a]$P < 0.01$ compared with OVX. [b]$P < 0.01$ compared with sham controls.

The ribonuclease protection analysis of PTHrP mRNA supported the results obtained by RT-PCR (Fig. 2). In the OVX+E40 group, PTHrP mRNA was significantly increased (0.984 ± 0.506 as the ratio of PTHrP/GAPDH mRNAs expression in arbitrary units) compared to sham-operated (0.213 ± 0.078, $P < 0.01$) or untreated OVX animals (0.110 ± 0.051, $P < 0.05$), but no significant difference was observed for OVX+E4 animals (0.420 ± 0.140).

We tried to evaluate iPTHrP expression levels using a human-PTHrP (1-34) IRMA kit in cytosol extracts of the kidneys, but values were not detectable in a sufficient number of samples to elaborate statistical analysis, probably due to low levels of the protein.

PTH/PTHrP Receptor Gene Expression and Adenylate Cyclase Activity Are Not Affected by Ovariectomy or Estrogen Repletion

PTH/PTHrP receptor mRNA and adenylate cyclase activity were not affected by ovariectomy or estrogen replacement treatment with either 4 or 40 µg E2/kg/day. RT-PCR analysis of

PTH/PTHrP receptor mRNA failed to show any difference among the groups (Fig. 3). In situ hybridization (Fig. 4) revealed no differences in renal distribution of receptor transcripts; the mRNAs were located closely in the renal cortex in glomerular epithelial cells and in proximal, distal, and collecting tubule cells. We also measured PTH/PTHrP receptor activity in renal cortical membranes, and, after stimulation of adenylate cyclase activity with PTH (10^{-6} M), cAMP accumulation was unaffected by ovariectomy or E2 treatment (Fig. 5).

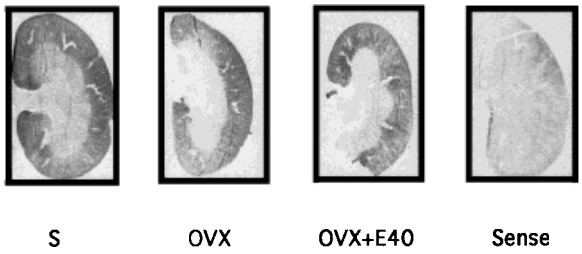

Fig. 4. PTH/PTHrP receptor mRNA analysis by in situ hybridization in the kidney. Representative sections of each group were hybridized with a specific antisense ^{35}S-labeled riboprobe, and labeling was evaluated after exposure for 48 h for autoradiography. Specific labeling was attested by hybridization with a sense ^{35}S-labeled riboprobe. OVX, ovariectomized; OVX+E4, ovariectomized receiving 4 µg E2/kg/day; OVX+E40, ovariectomized receiving 40 µg E2/kg/day; S, sham-operated.

DISCUSSION

In the present ovariectomized rat model, we found that, although PTHrP gene expression was unaffected by estrogen deficiency induced by ovariectomy, it was increased by pharmacological 17β-estradiol supplementation, whereas PTH/PTHrP receptor mRNA levels or adenylyl cyclase-coupled activation were not affected by ovariectomy or estrogen treatment. Calcemia, and in a more discrete manner circulating PTH levels, were modified as a function of estrogen status. Other studies performed in rats have suggested that estrogens may regulate PTH and/or calcium homeostasis, but the results of these studies are controversial: some authors have failed to observe any difference in PTH levels after oophorectomy, with a significant increase in urine calcium [Dick et al., 1996]; others found a nonsignificant fall in PTH mRNA levels after ovariectomy and a significant increase after estrogen treatment [Naveh-Many et al., 1992]. We found a significant reduction in plasma PTH after ovariectomy ($P < 0.05$) and a parallel decrease in plasma calcium ($P < 0.01$) even if these changes have only minor physiological significance. Treatment with 4 or 40 µg E2/kg/day after ovariectomy restored plasma PTH and calcium levels to control values.

Interestingly, estrogen replacement therapy modified PTHrP mRNA levels in the kidney. In our study, we observed no difference in PTHrP mRNA expression in OVX or in OVX+E4 groups but a two- to fivefold increase in PTHrP mRNA expression after substitutive treatment with 40 µg E2/kg/day (OVX+E40). The lack of effect of the lowest dose might be an oddity, because

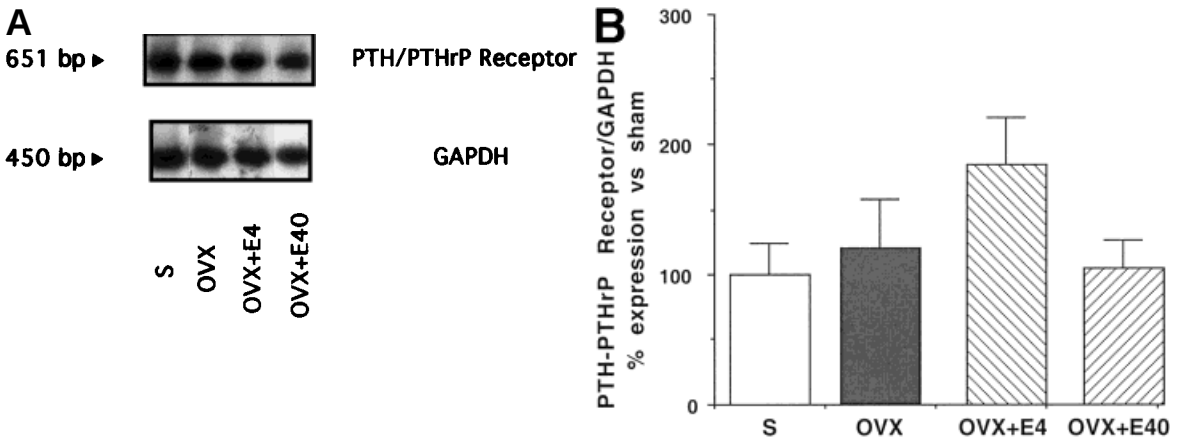

Fig. 3. Semiquantitative RT-PCR analysis of PTH/PTHrP receptor mRNA expression in the kidney. bp, base pairs; OVX, ovariectomized (n = 8); OVX+E4, ovariectomized receiving 4 µg E2/kg/day (n = 5); OVX+E40, ovariectomized receiving 40 µg E2/k/day (n = 10); S, sham-operated (n = 9). **A:** Southern blot analysis of PCR products was realized as decribed in Materials and Methods. One representative sample is shown for each group. Primers were used to amplify a 651 bp PTH/PTHrP receptor fragment and a 450 bp GAPDH fragment. **B.** Quantitative results are presented as the ratio of PTH/PTHrP receptor to GAPDH (internal standard). Results shown are mean ± SEM of the percentage of expression from sham controls.

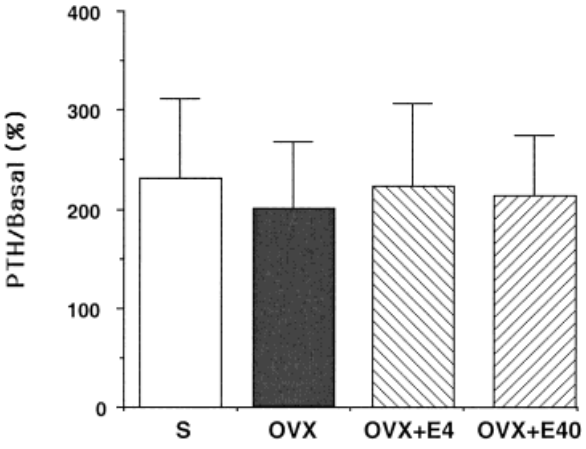

Fig. 5. Adenylate cyclase activity of PTH/PTHrP receptor in the kidney. Cyclic AMP accumulation after stimulation with PTH (10^{-6} M) was evaluated as described in Materials and Methods. Quantitative results are presented as the percentage of cAMP accumulation after stimulation with PTH compared to basal cAMP accumulation after stimulation with acetic acid (10^{-3} M). Results shown are mean ± SEM of the percentage for each group (n = 10).

when we analysed the results by the Student's *t*-test OVX rats were significantly lower than OVX+E4, whereas no significance was noted by one-way ANOVA. The fact that only the highest dose of E2 induces a significant increase of PTHrP mRNA expression may also be explained by the estrogen receptor status of the kidney. The effect of estrogen is highly dependent on the number of receptors expressed and thus on the tissue studied [Orloff et al., 1989]. Contrarily to the uterus, which is considered an estrogen receptor–rich tissue, the kidney [Hagenfeldt and Erikson, 1988] is a secondary target tissue for estrogens [Katzenellenbogen et al., 1993] with low receptor content. For instance, estrogen effects on bone resorption were observed for doses about 100 µg E2/kg/day [Chow et al., 1992; Shen et al., 1993]. Various studies have previously shown that PTHrP mRNA expression is dependent on E2 status both in vitro in primary myometrial cells and a rat pituitary cell line [Suva et al., 1991; Weir et al., 1991; Holt et al., 1994] and in vivo in the uterus [Thiede et al., 1991], but the mechanism of PTHrP regulation by estrogens has not been elucidated yet. Although there is no evidence of an estrogen-responsive element in the PTHrP gene, it has been suggested that, as the effects of estrogen were blocked by dexamethasone [Suva et al., 1991], the responsiveness of the PTHrP gene to estrogen may be due to interactions with the previously described glucocorticoid-regulation pathway [Ikeda et al., 1989]. These results raise once more the possibility of a local PTHrP gene regulation by estrogens in the kidney.

As PTHrP acts as an autocrine-paracrine factor through the PTH/PTHrP receptor, we asked whether this receptor was also affected by OVX or E2 substitutive treatment. RT-PCR analysis, in situ hybridization, and cAMP assay failed to show any modification of the mRNAs levels, renal distribution, and adenylyl cyclase activity of the PTH/PTHrP receptor by ovariectomy and estrogen substitutive treatment. A dose response to PTH stimulation might have been more informative to observe changes in sensibility even if the regulation of the PTH-mediated cAMP response by estrogens has been reported only in bone cells [Fukayama and Tashjian, 1989; Kaji et al., 1996]. So, estrogen's effects on the kidney seem to be mediated by a regulation of PTHrP and not by changes of the PTH/PTHrP receptor gene expression.

The precise role of PTHrP in the kidney is not well defined. A classical PTH-like action of PTHrP, mediated via the PTH/PTHrP receptor, is to increase the renal tubular reabsorption of calcium [Strewler and Nissenson, 1994]. A recent study has shown that estrogen also enhances the calbindin-D_{28k} mRNA expression in the rat kidney [Criddle et al., 1997] and proposes that the kidney may participate in calcium homeostasis in particular after the menopause. It is likely to think that pharmacological actions of estrogen on the kidney may also be mediated through PTHrP and PTH/PTHrP receptor and lead to an increase in tubular reabsorption of calcium, limitating the renal leak of calcium observed in postmenopausal women [Nordin et al., 1991].

In conclusion, we found that renal PTHrP and PTH/PTHrP receptor mRNA expression was not modified by ovariectomy. However, estrogen replacement treatment significantly increased the renal PTHrP mRNA expression without evident changes in the renal PTH/PTHrP receptor mRNA expression and adenylyl cyclase activation. This study suggests that, in the kidney, pharmacological action of estrogen may be in part mediated by PTHrP. This supports the hypothesis that estrogen supplementation of postmenopausal women may inhibit renal calcium leak and prevent bone loss [Nordin et al., 1991] by promoting tubular calcium reabsorp-

tion through a local increase in renal PTHrP expression [Strewler and Nissenson, 1994]. Further in vitro studies to explore the PTHrP mRNA and protein expression in cultured renal cell lines will be helpful to understand the mechanisms of estrogens on PTHrP expression.

REFERENCES

Abou-Samra A-B, Jüppner H, Force H, Freeman MW, Kong XF, Schipani E, Ureña P, Richards J, Bonventre JV, Potts JT Jr, Kronenberg HM, Segre GV. (1992): Expression cloning of a parathyroid hormone/parathyroid hormone–related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol triphosphate and increases intracellular free calcium. Proc Natl Acad Sci U S A 89:2732–2736.

Adami S, Gatti D, Bertoldo F, Rossini M, Fratta-Pasini A, Zamberlan N, Facci E, Lo Cascio V (1992): The effects of menopause and estrogen replacement therapy on the renal handling of calcium. Osteoporosis Int. 2:180–185.

Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC (1994): Parathyroid hormone–related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol 126 6:1611–1623.

Chomiczynski P, Sacchi N (1987): Single-step of RNA isolation by acid guanidium thiocyanate-phenol-chloroforme extraction. Anal Biochem 162:156–159.

Chow J, Tobias JH, Colston KW, Chambers TJ (1992): Estrogen maintains trabecular bone volume in rat not only by suppression of bone resorption but also by stimulation of bone formation. J Clin Invest 89:74–78.

Criddle RA, Zheng MH, Dick IM, Callus B, Prince RL (1997): Estrogen responsiveness of renal calbindin-D_{28k} gene expression in rat kidney. J Cell Biochem 65:340–348.

Dick LM, StJohn A, Heal S, Prince RL (1996): The effect of estrogen deficiency on bone mineral density, renal calcium and phosphorus handling and calciotropic hormones in the rat. Calcif Tissue Int 59:174–178.

Fukayama S, Tashjian AH (1989): Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 124:397–401.

Garcia-Ocaña A, de Miguel F, Peñaranda C, Albar JP, Sarasa JL, Esbrit P (1995): Parathyroid hormone–related protein is an autocrine modulator of rabbit proximal tubule cell growth. J Bone Miner Res 10:1875–1884.

Hagenfeldt Y, Eriksson HA (1988): The estrogen receptor in the rat kidney. Ontogeny, properties and effects of gonadectomy on its concentration. J Steroid Biochem 31:49–56.

Holt EH, Lu CE, Dreyer B, Dannies PS, Broadus AE (1994). Regulation of parathyroid hormone–related peptide gene expression by estrogen in GH_4C_1 rat pituitary cells has the pattern of a primary response gene. J Neurochem 62:1239–1246.

Ikeda K, Lu C, Weir EC, Mangin M, Broadus AE (1989): Transcriptional regulation of the parathyroid hormone–related peptide gene by glucocorticoids and vitamin D in a human C-cell line. J Biol Chem 264:15743–15746.

Jüppner H, Abou-Samra A-B, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LF Jr., Hock J, Potts JT Jr., Kronenberg HM, Segre GV (1991): A G protein–linked receptor for parathyroid hormone and parathyroid hormone–related peptide. Science 254:1024–1026.

Kaji H, Sugimoto T, Kanatani M, Nasu M, Chihara K (1996): Estrogen blocks parathyroid hormone (PTH)–stimulated osteoclast-like cell formation by selectively affecting PTH-responsive cyclic adenosine monophosphate pathway. Endocrinology 137:2217–2224.

Karaplis AC, Yasuda T, Hendy GN, Goltzman D and Banville D (1990): Gene encoding parathyroid hormone–like peptide: Nucleotide sequence of the rat gene and comparison with the human. Mol Endocrinol 4:441–446.

Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VLJ, Kronenberg HM, Mulligan RC (1994): Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone–related peptide gene. Genes Dev 8:277–289.

Katzenellenbogen JA, Mathias CJ, Vanbrocklin HF, Brodack JW, Welch MJ (1993): Titration of the in vivo uptake of 16a-[18F]fluoroestradiol by target tissues in the rat: competition by tamoxifen, and implication for quantitating estrogen receptors in vivo and the use of animals models in receptor-binding radiopharmaceutical development.Nuclear Medicine and Biology 20:735–745.

Kramer S, Reynolds FH Jr, Castillo M, Valenzuela DM, Thorikay M, Sorvillo JM (1991): Immunological identification and distribution of parathyroid hormone–like protein polypeptides in normal and malignant tissues. Endocrinology 128:1927–1937.

Lee K, Deeds JD, Segre GV (1995): Expression of parathyroid hormone–related peptide and its receptor messenger ribonucleic acids during fetal development of rats. Endocrinology 136:453–463.

Marx SJ, Fedak SA, Aurbach GD (1972): Preparation and characterization of a hormone responsive renal plasma membrane fraction. J Biol Chem 247:6913–6918.

Naveh-Many T, Almogi G, Livni N, Silver J (1992): Estrogen receptors and biologic response in rat parathyroid tissue and C cells. J Clin Invest 90:2434–2438.

Nordin BEC, Need AG, Morris HA, Horowitz M, Robertson WG (1991) Evidence for a renal calcium leak in postmenopausal women. J Clin Endocrinol Metab 72:401–407.

Nyby MD, Hino T, Berger ME, Ormsby BL, Golub MS, Brickman AS (1995): Desensitization of vascular tissue to parathyroid hormone and parathyroid hormone–related protein. Endocrinology 136:2497–2504.

Orloff JJ, Wu TL, Stewart AF (1989): Parathyroid hormone–like proteins: biochemical responses and receptor interactions. Endocr Rev 10:476–495.

Prince RL (1994): Counterpoint: Estrogen effects on calciotropic hormones and calcium homeostasis. Endocr Rev 15:301–309.

Shen V, Dempster DW, Birchman R, Xu R, Lindsay R (1993): Loss of cancellous bone mass and connectivity in ovariectomized rats can be restored by combined treatment with parathyroid hormone and estradiol. J Clin Invest 91:2479–2487.

Shew RL, Yee JA, Kliewer DB, Keflemariam YJ, McNeill DL (1991): Parathyroid hormone–related protein inhibits stimulated uterine contraction in vitro. J Bone Miner Res 6:955–959.

Soifer NE, Van Why SK, Ganz MB, Kashgarian M, Siegel NJ, Stewart AF (1993): Expression of parathyroid hormone–related protein in the rat glomerulus and tubule during recovery from renal ischemia. J Clin Invest 92:2850–2857.

Strewler GJ, Nissenson RA (1994): Skeletal and renal actions of parathyroid hormone-related protein. In Bilezikian JP, Levine MA, Marcus R (eds): "The parathyroids." New York: Raven Press, Ltd., pp 311–320.

Suarez F, Lebrun J-J, Lecossier D, Escoubet B, Coureau C, Silve C (1995): Expression and modulation of the parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acid in skin fibroblasts from patients with type Ib pseudohypoparathyroidism. J Clin Endocrinol Metab 80:965–970.

Suva LJ, Winslow GA, Wettenhall REH, Hammonds RG, Moseley JM, Dieffenbach-Jagger H, Rodda CP, Kemp BE, Rodriguez H, Chen EY, Hudson PJ, Martin TJ, Wood W (1987): A parathyroid hormone–related protein implicated in malignant hypercalcemia: Cloning and expression. Science 237:893–896.

Suva LJ, Gillespie MT, Center RJ, Gardner RM, Rodan GA, Martin TJ, Thiede MA (1991): A sequence in the human PTHrP gene promoter responsive to estrogen. J Bone Mineral Res 6(suppl 1):S196 (abstract).

Thiede MA, Harm SC, Hasson DM, Gardner RM (1991): In vivo regulation of parathyroid hormone–related peptide messenger ribonucleic acid in the rat uterus by 17β-estradiol. Endocrinology 128:2317–2323.

Ureña P, Kong X-F, Abou-Samra A-B, Jüppner H, Kronenberg HM, Potts JT Jr, Segre GV (1993): Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 133:617–623.

Weir E, Daifotos A, Dreyer B, Burtis W, Broadus A (1991): Estrogen-responsive expression of the parathyroid hormone–related peptide gene by cultured primary myometrial cells. J Bone Miner Res 6(suppl 1):S233 (abstract).

Yates AJP, Gutierrez GE, Smolens P, Travis PS, Katz MS, Aufdemorte TB, Boyce BF, Hymer TK, Poser JW, Mundy GR (1988): Effects of a synthetic peptide of a parathyroid hormone–related protein on calcium homeostasis, renal tubular calcium reabsorption, and bone metabolism in vivo and in vitro in rodents. J Clin Invest 81:932–938.