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OESTROGEN ADMINISTRATION AND THE EXPRESSION OF THE KALLIKREIN GENE FAMILY IN THE RAT SUBMANDIBULAR GLAND

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Summary—Using a series of oligonucleotide probes (18–21 mers) specific for members of the rat kallikrein/tonin (arginyl-esteropeptidase) gene family (PS, S1, S2, S3, K1, P1), we have shown by Northern blot analysis that all six genes are expressed in the submandibular gland (SMG), with PS (true kallikrein) the most abundant in both male and female rats. Though female levels of PS mRNA are similar to that in the male, levels of mRNA from both the kallikrein-like (S1, K1, P1) and tonin (S2)/tonin-like (S3) genes are all substantially lower in the female than in the male rat. In contrast with the oestrogen dependence of anterior pituitary kallikrein (PS) gene expression, oestrogen administration (6 μg/day for 8 days) to castrate male or female rats is without effect on PS or S1, S2, S3, K1, P1 mRNA levels in the SMG. These findings suggest a tissue-specificity in the oestrogen regulation of true kallikrein gene expression in the two tissues. In intact male rats, oestrogen administration lowers SMG levels of S1, S2, S3, K1, and P1 but not PS mRNA to castrate levels, presumably by suppression of the pituitary/gonadal axis, consistent with the previously reported androgen dependence of SMG expression of these genes with the exception of PS.

INTRODUCTION

The glandular kallikreins are a family of arginyl-esteropeptidase enzymes, some of which have been implicated in the processing of polypeptide precursors to their bioactive forms [reviewed in 1–3]. In the rat, this family probably consists of between 11 and 20 genes [4–6]. Of these, the mRNA sequences for six distinct rat kallikrein family gene members (PS, S1, S2, S3, K1 and P1) have been derived from various tissue (pancreatic, submandibular gland, kidney and prostatic) cDNA libraries, [7–9; J. Brady and R. MacDonald, manuscript in preparation). These, encode true kallikrein (PS), tonin (S2) and four other similar but distinct candidate arginyl esteropeptidases. These latter genes have been designated kallikrein-like (S1, K1, P1) or tonin-like (S3) in terms of their amino acid sequence, although specific enzymatic functions are as yet unknown. In addition, these six genes, all of which are expressed in the submandibular gland (SMG), have been shown to be variously expressed in other tissues (PS—pancreas, kidney, anterior pituitary; K1—kidney; S3—prostate; P1—prostate [7–12; J. Brady and R. MacDonald, manuscript in preparation]).

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The major site of kallikrein gene expression in the rat is the submandibular gland (SMG) [4]. In previous studies on the androgen dependence of kallikrein gene expression, we have shown that levels of PS gene expression in SMG are unaffected by castration; in contrast, mRNA levels for the other five arginyl esteropeptidase genes (S1, S2, S3, K1 and P1) fall 40–60% after castration, and are unaffected or only partially restored by androgen replacement or oestrogen administration [10]. We have also shown, using both a rat pancreatic kallikrein cDNA probe known to cross-hybridize with other gene family members [7] and gene-specific oligonucleotide probes [12], that the levels of rat AP kallikrein mRNA, previously reported to be PS [11] are similarly regulated by oestrogens [12, 13].

In the present study we have explored the possibility of oestrogen effects both on PS gene expression, and that of the other kallikrein family members, in the male and female rat SMG.

EXPERIMENTAL (WITH METHODOLOGY)

Animal experimental protocols

Sprague–Dawley rats weighing 120–160 g, from a pathogen-free colony bred in the Central Animal House of Monash University, were used in all experiments. Rats were maintained on water and standard rat chow *ad libitum*.

Table 1. Oligonucleotide probes specific for different rat kallikrein genes

Gene	mRNA Sequence	No. of differences with[a]					
		PS	S1	S2	S3	K1	P1
PS	CAT CAC ACC TGA CGG ATT GGA(ex3/4)	—	10	11	12	10	6
S1	GGG AAT TCC CTG ATG ATC(ex4)	4	—	7	6	0	1
	ACC CCT CTG AGT GGGA(ex3/4)	9	—	3	3	6	8
S2	CCA CTC ATC GTG ACG AAT	11	6/14	—	5	7	8
S3	ATC CCA GTC TTC ATG AGG AAC	7	4/14	5	—	4	10
K1	AAA CCA TTC CTC ATG AGG AAC	7	3/14	7	4	—	8
P1	TCG GAC ATC ATA AAG AAC[c]	5	3/10	8	6	7	—

[a]Number of nucleotide differences between the mRNA sequence from which the oligonucleotide probe was derived and the corresponding region in the other 5 mRNA sequences. [b]Number of nucleotide differences/number of nucleotides sequenced over this region (S1 is a partial cDNA only). [c]The sequence of P1 shown here is that of the probe synthesized, not of P1 mRNA, which has the first two nucleotides CT rather than TC. Given the hybridization controls within each experiment, we believe that the patterns shown are specific for P1, and that the probe should be operationally considered a 16 mer rather than an 18 mer.

Male or female Sprague–Dawley rats were castrated under light ether anaesthesia. Intact or castrate male rats were treated with vehicle, 6 μg 17β-oestradiol-3-benzoate (E_2, Sigma, St. Louis, Mo.) or 1 mg dihydrotestosterone (DHT, Sigma) in 0.1 ml maize oil by daily i.m. injection for 8 days. Intact or ovariectomized female rats were treated with vehicle or 6 μg E_2 daily for 7 days. Animals were killed by decapitation, and the SMG dissected, snap frozen in liquid nitrogen and stored at -70°C until processed.

Kallikrein mRNA analysis

Total RNA was isolated from whole tissues by the method of Chirgwin *et al.*[14], and Northern blotting was performed as previously described [15]. Briefly, 2–25 μg total RNA was denatured in 1 M glyoxal/50% dimethylsulfoxide, electrophoresed in a 1.2% agarose gel and passively transferred to Hybond (Amersham) nylon membranes by capillary blotting. The nylon membranes were baked at 80°C for 1 h, u.v. crosslinked for 10 min and prehybridized for 4–24 h at 42°C in Northern hybridization buffer [50% formamide, 5 × SSC (1 × SSC is 0.15 M sodium chloride–0.15 M sodium citrate, pH 7.4), 50 mM sodium phosphate (pH 8.0), 10 × Denhardt's solution, 100 μg/ml herring sperm DNA] for subsequent hybridization with cDNA or long (30 mer) oligonucleotide probes. For hybridization with shorter (18–21 mer) oligonucleotide probes, blots were also prehybridized for 4–24 h at 42°C in 5 × SSC, 50 mM sodium phosphate (pH 8.0), 10 × Denhardt's solution, 0.1% $NaDodSO_4$, 0.01% sodium pyrophosphate and 100 μg/ml herring sperm DNA. Blots were hybridized with the appropriate ^{32}P-labelled probe (1 × 10^6 cpm/ml) for 24–48 h at 37°C (18–21 mer) or 42°C (30 mer, cDNA), washed in 0.1 × SSC, 0.1% $NaDodSO_4$ at room temperature, and then at 37°C (18–21 mer) or 50°C (30 mer, cDNA).

Following autoradiography, densitometry (ISCO gel scanner 1312) was used to assess experimental changes observed in the hybridization of specific gene probes relative to levels with the control probe, a synthetic 30 mer for rat 18S ribosomal RNA. At least two different exposures of the same autoradiograph were routinely evaluated in order to ensure that the specific mRNA levels measured were in a linear range of silver grain deposition on the autoradiographic film. For rehybridization with a different probe, blots were boiled for 3 min in sterile, deionized, distilled water and exposed to Kodak XAR-5 film overnight, to check completeness of probe removal.

cDNA and oligonucleotide probes

A 620 b.p. rat pancreatic kallikrein cDNA [7] was used as a general kallikrein probe. Oligonucleotide probes (18–21 mer) with varying specificities for the previously described [8] rat kallikrein genes (PS, S1, S2 and S3) were derived from one or more of 3 relatively dissimilar regions of these genes. Oligonucleotide sequences and relative specificities are described in Table 1. In addition, oligonucleotide probes specific for two more rat kallikrein genes recently cloned and sequenced from kidney (K1) and prostate (P1) cDNA [9; J. Brady and R. MacDonald, manuscript in preparation] have also been included. To ensure hybridization and wash conditions were appropriately stringent to distinguish the different kallikrein gene-specific mRNAs, dot blots of 5 ng denatured cloned cDNAs (PS, S1, S2, S3, P1 and K1) were included in each Northern hybridization with the specific oligomers.

As a control for RNA species not expected to change under the experimental conditions studied, a rat 18S ribosomal RNA (CGG CAT GTA TTA GCT CTA GAA TTA CCA CAG) oligonucleotide probe (30 mer) [16] was used to assess the amount and integrity of total RNA loaded onto each gel. Although many other investigators use actin or tubulin as internal control probes, the levels of both these mRNA have been shown to change under certain experimental conditions [17], and there are large between-tissue variations in abundance [18]. In our hands, 18S ribosomal RNA levels have proved to be the better internal control, both in terms of relative abundance in all tissues and the lack of change in levels for all experimental situations so far studied. cDNA probes were labeled with α^{32}P-dCTP (BRESATEC, Adelaide, South Australia, 1800 Ci/mmol) to a specific activity of 10^8–10^9 cpm/μg by the method of Feinberg and Vogelstein [19]. Oligonucleotide probes were end-labelled

with γ^{32}P-ATP (BRESATEC, Adelaide, South Australia, 2000 Ci/mmol) to a similar specific activity.

RESULTS

In order to assess the direct oestrogen dependence, if any, of SMG arginyl-esteropeptidase gene expression in both the male and female rat, several Northern blots of SMG RNA from intact, castrate and oestrogen-treated animals were hybridized with both the kallikrein cDNA probe and the specific oligomers for each of the six identified rat kallikrein genes (PS, S1, S2, S3, K1, P1). In addition, all blots were stripped and rehybridized with the rat 18S ribosomal probe. To assess the possibility of indirect effects of oestrogen, via suppression of the pituitary-gonadal axis, intact male rats were also treated with the same doses of oestrogen; as an additional internal control, castrate male rats were treated with dihydrotestosterone, a non-aromatizable androgen. Representative Northern blot analyses of SMG mRNA from male rats are shown as Figs 1 and 2.

In Fig. 1, blots were hybridized with the pancreatic kallikrein cDNA, with the PS (true kallikrein) oligomer, or with the probe for 18S ribosomal RNA. There are no marked differences in loading, as assessed by the 18S panel, nor in mRNA levels determined at either exposure time for either probe. In contrast, the marked effects of oestrogen administration to intact male rats and of castration can be seen in Fig. 2. For all five of these genes (S1, S2, S3, K1 and P1), expression is clearly altered by oestrogen administration to intact male rats and in a largely androgen-reversible fashion by castration.

Densitometric data from specific hybridization studies ($n = 2$–5) are shown for both male and female rats in Fig. 3 and are expressed relative to the 18S densitometric profile for each individual blot. Levels in the intact male rat are arbitrarily set at unity. SMG levels of kallikrein mRNA assessed by hybridization with the general kallikrein cDNA probe were unaffected by oestrogen (6 μg/day for 8 days) administration; similarly, levels did not change with castration or steroid administration to the castrate male rat (Fig. 3, upper left panel), as has been previously reported [10]. Female SMG kallikrein mRNA levels were similar to those in the male, and unchanged by ovariectomy for 8 days or E_2 replacement (6 μg/day for 8 days) to the ovariectomized rat (Fig. 3, upper right panel).

PS (true kallikrein) mRNA levels were similarly unaffected by any of these treatments (second panel), mirroring those seen with the kallikrein cDNA, which thus presumably reflects the predominance of PS mRNA in the SMG compared with the mRNA levels of the other members of this family (S1, S2, S3, K1, P1) so far identified. The SMG levels of S1, K1, P1 (kallikrein-like), S2 (tonin) or S3 (tonin-like) mRNA in the male rat all appeared lower (with the possible exception of P1) after E_2 administration; the extent of the fall was similar to that seen after castration, where again the P1 response was the most variable (Fig. 3, middle left panels). In the castrate male rat, the administration of DHT increased expression of S1, K1, S2 and S3 towards, but not to, levels seen in the intact male rat; no change was seen in P1 mRNA after DHT, nor in SMG levels of mRNA encoded

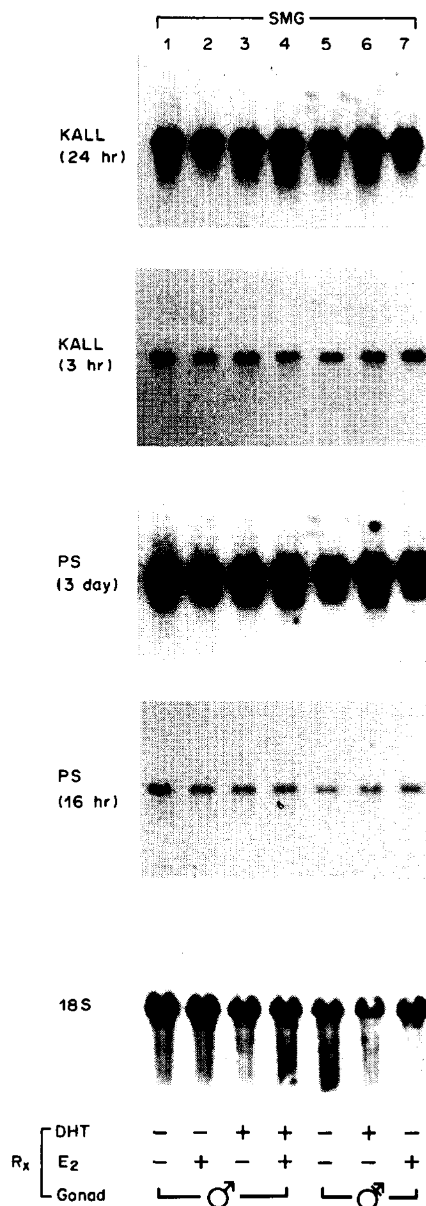

Fig. 1. Sequential hybridization of ^{32}P-labelled cDNA for rat pancreatic kallikrein (KALL), of an oligonucleotide probe specific for true kallikrein (PS) and of a rat 18S ribosomal RNA oligonucleotide probe to a Northern blot of 20 μg total RNA from SMG of intact males (lane 1), intact males given oestradiol 6 μg/day for 7 days (lane 2), castrate males (lane 3), castrate males given dihydrotestosterone 1 mg/day for 7 days (lane 4), or castrate males given oestradiol. Two different autoradiographic exposures are shown for KALL and PS. Length of time for autoradiography with Fuji RX film is indicated in parenthesis. Autoradiography for 18S was 16 h with Agfa Ospray RPC film.

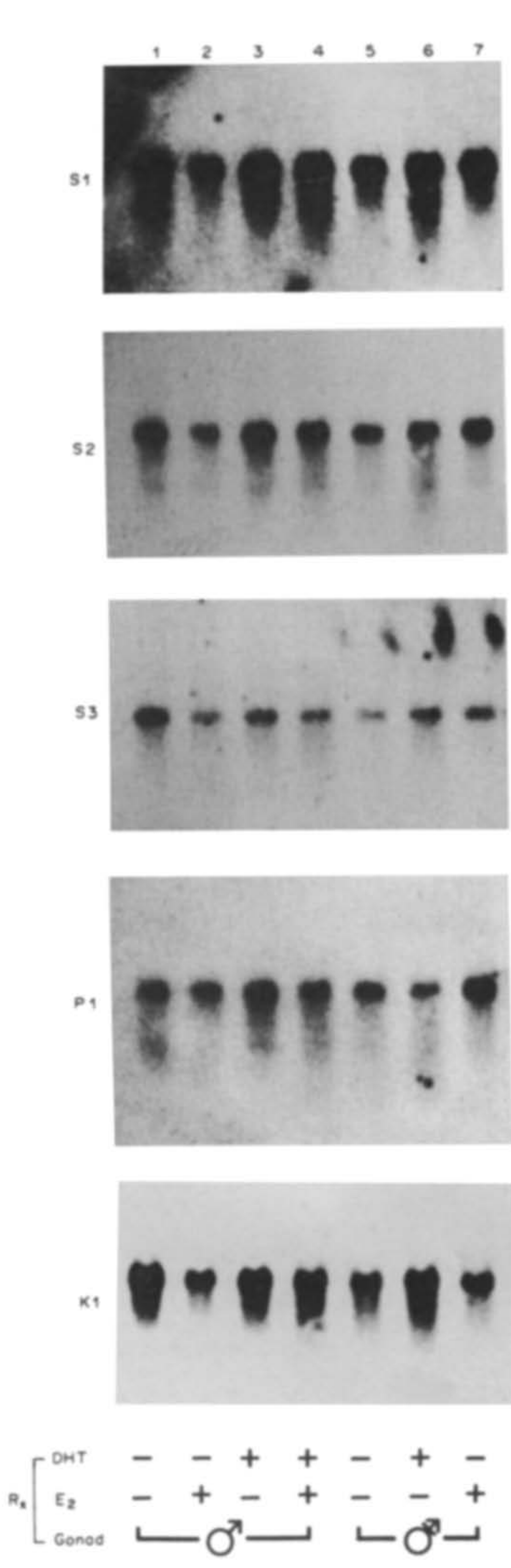

Fig. 2. Sequential hybridization of five oligonucleotide probes specific for kallikrein-like (S1, K1, P1), tonin (S2), and tonin-like (S3) members of the kallikrein gene family: lanes as for Fig. 1. Autoradiography was for 2 days (S2), 3 days (P1, K1) 7 days (S3) and 8 days (S1) with Fuji RX film.

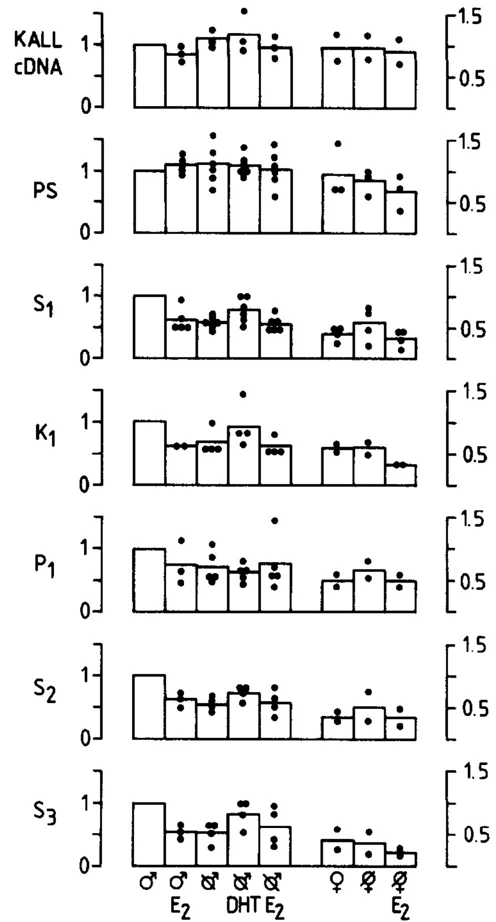

Fig. 3. Levels of kallikrein (KALL cDNA, PS), kallikrein-like (S1, P1, K1), tonin (S2) and tonin-like (S3) mRNA in the rat SMG. Densitometric analysis of 2–6 Northern blots of 20 μg total SMG RNA from intact male (♂) rats with or without oestradiol (E_2) treatment (6 μg/day for 7 days), castrate male (castrate ♂) rats, castrate males treated with dihydrotestosterone (DHT; 1 mg/day for 6 days) or E_2, intact female rats (♀), castrate females (castrate ♀), or castrate females treated with E_2. All values are expressed relative to those obtained with the control 18S ribosomal probe, and values for intact males are arbitrarily assigned as unity for each gel. Individual values for individual experiments (n = 2–6) are shown, plus mean values as a bar.

by all five genes, after oestrogen administration to castrate rats.

Neither ovariectomy nor the administration of E_2 (6 μg/day) for 8 days had any effect on the levels of S1, S2, S3, K1 or P1 mRNA in the female SMG (Fig. 3, right, middle panels). Of interest, however, is the substantial sex difference in the expression of these five genes in this tissue, in that female mRNA levels are very much lower than those in the male SMG. This difference is not seen when the general kallikrein cDNA probe is used (Fig. 3, upper right panel), again reflecting the much less marked sex differences in the SMG levels of the more abundant PS mRNA.

DISCUSSION

We have previously demonstrated that PS (true kallikrein) gene expression, as determined by hybridization with both PS cDNA and gene-specific oligonucleotide probes, is regulated by oestrogen in the rat anterior pituitary [12, 13, 20]. Given this finding, the lack of an equivalent regulation of PS mRNA levels in the SMG, particularly in the female in the present study, was somewhat unexpected. This may reflect the absence of oestrogen receptors in those cells of the SMG in which the PS gene is expressed; alternatively, the oestrogen responsiveness of PS gene expression in the rat pituitary may be secondary to other hormonally-induced cellular events, or reflect some other tissue-specific mechanism.

We have also previously shown that PS, true kallikrein, is the most abundant member of the kallikrein gene family expressed in the male SMG and is not regulated by androgens, and have reported the partial androgen dependence of the less abundant members (S1, S2, S3, K1, P1) of this argingylesteropeptidase family in the male SMG [10]. In the present study, we have extended these observations to show that E_2 administration (6 μg/day for 8 days) to the intact male rat elicits a fall in SMG mRNA levels of S1, S2, S3, K1 and perhaps of P1, but not of PS. Since the fall in mRNA levels (25–50%) observed for each individual gene is comparable to that seen with castration, we presume that E_2 is not directly influencing SMG kallikrein gene expression, but rather indirectly by suppressing pituitary gonadotrophins, thus altering the androgen status of the animal.

The observation that SMG levels of PS mRNA are not different between male and female rats is in keeping with the lack of effect of androgens on PS gene expression [10]. Similarly, the degree of sex difference in SMG mRNA levels (female 25–60% those of male) for the other five arginylesteropeptidase genes (S1, S2, S3, K1, P1) presumably reflects the androgen dependence of these genes in this tissue.

Kallikrein mRNA [10], immunoreactivity and enzyme activity [21] have all been reported to be 2-fold higher in the male than female SMG. Although this difference is not major, it presumably reflects the expression of the sex-dependent but less abundant kallikrein-like genes (S1, K1 and P1) over and above that of the PS gene. The more marked sex difference in tonin (S2)/tonin-like (S3) SMG mRNA levels is also consistent with the 10-fold higher ir-tonin levels reported in the male SMG than in the female [22]. These results are also similar to those found in the mouse SMG, where the mRNA levels of true kallikrein (MGK6) were shown not to be different between sexes, whereas mRNA levels for other members of the mouse kallikrein gene family (MGK3, MGK4) were much higher in male than female animals [23].

Taken together, these findings serve to emphasize a tissue-specific diversity in hormonal regulation of this gene family. Whether the mechanism of regulation is a primary transcriptional response, perhaps modulated by tissue-specific factors or secondary to hormonally induced cell/tissue specific events, is yet to be established.

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