Tissue-specific regulation of the expression of rat kallikrein gene family members by thyroid hormone

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We have altered the thyroid hormonal status of both male and female rats and examined the expression of six functional members of the rat kallikrein gene family (PS, S1, S2, S3, K1 and P1) in the submandibular gland (SMG), kidney, prostate, testis and anterior pituitary gland (AP) of these animals. On Northern-blot analysis with gene-specific oligonucleotide probes, the steady-state mRNA levels of S1, S2, S3, K1 and P1 were all dramatically altered in the SMG of male and female rats treated with propylthiouracil (PTU; 100 mg/litre of drinking water) or thyroxine (T_4 ; 10 μ g/100 mg body wt.) for 3 weeks. The SMG mRNA levels of these five genes were all lowered (30–90%) in hypothyroid (PTU-treated) male and female rats and elevated (1.4–4-fold, male; 1.5–11-fold, female) in the hyperthyroid (T_4 -treated) and PTU/ T_4 -treated animals. In contrast, PS (true kallikrein) mRNA levels in the male or female SMG or kidney were essentially unchanged. K1 mRNA levels in the kidney were considerably less responsive to thyroid status than those in the SMG. Changes in S3 and P1 mRNA levels in the prostate were also variable, but essentially unaffected by these treatments. AP PS mRNA levels were also unaffected by changes in thyroid-hormonal status, as were levels of a novel P1-like mRNA in the testis. In summary, these studies demonstrate that the same kallikrein gene family member(s) may be differentially regulated by thyroid hormones in the rat SMG, kidney, prostate and pituitary, and thus further extend the concept of tissue-specific expression and hormonal regulation of the kallikrein gene family in the rat.

INTRODUCTION

The glandular or tissue kallikreins are a subgroup of serine proteinases, many of which have been shown to be involved in the post-translational processing of polypeptide precursors to their bioactive forms (reviewed in [1–4]). The tissue kallikreins are encoded by a multigene family which, in the rat, is thought to consist of 11–20 genes [5–7]. 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submandibular gland, kidney and prostatic) cDNA libraries [8–10; J. M. Brady, D. R. Wines & R. J. MacDonald, unpublished work]. PS was shown to encode true kallikrein and S2 tonin; S1, K1 and P1 were designated 'kallikrein-like' and S3 designated 'tonin-like' with respect to their amino acid sequence, although their specific enzymic function is 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 in pancreas, kidney, anterior pituitary; K1 in kidney; S3 in prostate; P1 in prostate [8–14; J. M. Brady, D. R. Wines & R. J. MacDonald, unpublished work]).

The hormonal regulation of the expression of these six kallikrein gene family members would also appear to be tissue-specific. We have previously demonstrated that, whereas S3, and to a lesser extent, P1 mRNA levels in the rat prostate and S1, S2, S3, K1 and P1 mRNA levels in the SMG are androgen-dependent, PS mRNA levels in the SMG and kidney, and K1 mRNA levels in the kidney are not so regulated [14]. In addition, we [13,15] and others [12,16–18] have demonstrated the oestrogen-dependence of kallikrein (PS) mRNA levels and kallikrein activity in the anterior pituitary gland (AP) of the rat.

Thyroxine (T_4) administration to intact female or castrated male rats has been shown to increase kallikrein enzyme- or immuno-reactivity [19] and mRNA levels [20], as detected by a tissue kallikrein cDNA probe, in the rat SMG. In addition, T_4

treatment has been shown to affect differentially the expression of the mouse kallikrein gene family members mGK3 (regulated) and mGK6 (unaffected) in the mouse SMG [21]. Thus, in the studies described below, we have sought to extend the above observations and further explore this tissue-specificity of regulation of the expression of various kallikrein gene family members in the rat. Using gene-specific oligonucleotide probes we have examined the steady-state mRNA levels for PS, S1, S2, S3, K1 and P1 in the SMG, kidney, prostate, testis and AP of the hypothyroid, hyperthyroid and propylthiouracil (PTU)/ T_4 -treated male and female rat.

MATERIALS AND METHODS

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*.

Male and female rats ($n = 6$ –10/group) were left untreated, injected with T_4 (Oroxine; Wellcome Australia Pty., Sydney, Australia) in saline (0.9% NaCl) suspension (10 μ g/100 g body wt.) or given PTU (Citag Pty., Sydney, Australia; 100 mg/litre of drinking water) or a combination of both for 3 weeks. Experiments on both male and female animals were repeated on an additional occasion, making a total of four experiments (two male, two female). Animals were killed by decapitation, the blood collected for serum 3,3',5-tri-iodothyronine (T_3)/ T_4 radioimmunoassay [22] and the relevant tissues (male: SMG, kidney, prostate, testis, AP; female: SMG, kidney, AP) dissected, snap-frozen in liquid N_2 and stored at -80°C until processed.

Kallikrein mRNA analysis

Total RNA was isolated from whole tissues pooled from each experimental group by the method of Chirgwin *et al.* [23].

Northern blotting was performed as previously described [13,14]. Briefly, 25 µg of total RNA was denatured in 1 M-glyoxal with 50% (v/v) dimethyl sulphoxide, electrophoresed in a 1.2%-agarose gel and passively transferred to Hybond (Amersham) nylon membranes by capillary blotting. An RNA ladder (BRL) was used as a molecular-mass marker in each gel. The nylon membranes were baked at 80 °C for 1 h, u.v.-cross-linked for 10 min and prehybridized for 4–24 h at 42 °C in 5 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.4)/50 mM-sodium phosphate, pH 8.0, 10 × Denhardt's solution/0.1% SDS/0.01% sodium pyrophosphate/herring sperm DNA (100 µg/ml) for hybridization with the shorter (18–21-mer) kallikrein gene-specific oligonucleotide probes. Blots were also prehybridized for 4–24 h at 42 °C or 50 °C in Northern hybridization buffer [50% formamide/5 × SSC/50 mM-sodium phosphate (pH 8.0)/10 × Denhardt's solution/herring sperm DNA (100 µg/ml)] for subsequent hybridization with the longer (30-mer) oligonucleotide or cDNA probes. Blots were hybridized with the appropriate ³²P-labelled probe (1 × 10⁶ c.p.m./ml) for 16–48 h at 30 °C (P1-specific oligomer), 37 °C (other kallikrein oligomers), 42 °C (cDNA) or 50 °C (18 S 30-mer), washed in 2 × SSC/0.1% SDS at room temperature, and then in 2 × SSC/0.1% SDS or 0.1 × SSC/0.1% SDS at 30/37 °C (18–21-mer) or 50 °C (cDNA, 30-mer).

After autoradiography, densitometry (ISCO gel scanner 1312/Hewlett-Packard HP3396A integrator) was performed to assess experimental changes observed in hybridization of specific kallikrein gene probes relative to levels of the control 18 S probe. Duplicate or triplicate Northern blots/tissue were generated for each experiment and hybridized with a selection of the kallikrein-gene-specific oligonucleotide probes as well as the 18 S control probe. The Northern blots shown in Figs. 1–4 are representative of these hybridizations. The fold or percentage changes referred to in the Results section were obtained from the densitometric values which were averaged for different exposures/Northern blots for each animal experiment and then expressed relative to 18 S-RNA levels. Peak areas for the control group were arbitrarily set at unity, and all values were expressed relative to control. 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

Oligonucleotide probes (18–21-mers) specific for the previously described [9,10; J. M. Brady, D. R. Wines & R. J. MacDonald,

unpublished work] rat kallikrein cDNA (PS, S1, S2, S3, K1 and P1) were derived from one or more of three relatively dissimilar regions of these cDNA sequences. Oligonucleotide sequences and relative specificities were described previously [14]. To ensure that hybridization and wash conditions were appropriately stringent to distinguish the different kallikrein gene-specific mRNAs, dot blots of 5 ng of denatured cloned cDNAs (PS, S1, S2, S3, P1 and K1) were included in each Northern hybridization with the specific oligomers.

A 600 bp rat prolactin [24] probe was used to detect AP prolactin mRNA. An additional oligonucleotide (30-mer) probe [14] for rat 18 S rRNA [25], an RNA species not expected to change under the experimental conditions studied, was used to assess the amount and integrity of total RNA loaded in each gel. cDNA probes were labelled with [α -³²P]dCTP (BRESATEC, Adelaide, South Australia, Australia; 1800 Ci/mmol) to a specific radioactivity of 10⁸–10⁹ c.p.m./µg by the method of Feinberg & Vogelstein [26]. The oligonucleotide probes were end-labelled with [γ -³²P]ATP (BRESATEC; 2000 Ci/mmol) to a similar specific radioactivity.

RESULTS

The thyroid-hormone status of the animals was assessed by their serum T₃ and T₄ levels, which are shown in Table 1. Rats treated with PTU showed marked decreases in both T₄ and T₃; those treated with T₄ showed increased T₄ levels compared with the control, though T₃ levels were increased only in male rats. Rats treated with both PTU and T₄ showed characteristically elevated levels of T₄ beyond those seen in the T₄-alone group, and T₃ levels below control, but higher than those in the PTU alone group, which is consistent with the 5'-monodeiodination *in vivo* of T₄ to T₃ by PTU in this group.

SMG mRNA levels, relative to 18 S ribosomal RNA levels, for five of the kallikrein gene family members expressed in this tissue (S1, S2, S3, P1, K1) fell markedly (30–70%) with PTU treatment in both male and female rats (Fig. 1). The SMG mRNA levels of these same five genes were increased on T₄ administration with or without PTU (Fig. 1). The magnitude of this increased response was variable for each gene product, with the change in female levels consistently higher (female, 1.5–12-fold; male, 1.4–4-fold), perhaps reflecting the very much lower female control levels. In contrast, PS (true kallikrein) mRNA levels in the male or female SMG are essentially unaffected by any of these treatments (Fig. 1).

As in the SMG, PS mRNA levels relative to 18 S RNA levels

Table 1. Serum T₃ and T₄ levels

Serum T₃/T₄ values (nmol/litre, means ± S.E.M.) for four separate experiments [two male (♂) and two female (♀)] are shown. The numbers of animals/group (n) are given in parentheses. T₃/T₄ measurements were done by radioimmunoassay as described in [25].

Thyroid hormone	Sex	n	Serum T ₃ or T ₄ level			
			Control	PTU	T ₄	PTU/T ₄
T ₃	♂	(8)	1.6 ± 0.1	0.7 ± 0.1	2.3 ± 0.5	1.2 ± 0.1
	♀	(10)	1.9 ± 0.1	0.7 ± 0.02	1.9 ± 0.2	1.3 ± 0.1
	♂	(10)	1.9 ± 0.1	0.8 ± 0.1	3.9 ± 0.4	1.5 ± 0.3
	♀	(10)	2.3 ± 0.1	0.7 ± 0.03	2.3 ± 0.1	1.2 ± 0.1
T ₄	♂	(8)	75 ± 5	20 ± 10	130 ± 19	284 ± 20
	♀	(10)	47 ± 5	4.2 ± 1.3	154 ± 11	281 ± 15
	♂	(10)	66 ± 2	6.5 ± 0.6	241 ± 35	368 ± 22
	♀	(10)	71 ± 5	6.2 ± 0.4	121 ± 8	186 ± 17

Fig. 1. Hybridization of ³²P-labelled PS; S1; S3; P1- and K1-kallikrein-gene-specific oligonucleotide probes and rat 18 S rRNA control probe to 25 µg of SMG total RNA from male (♂, lanes 1–4) or female (♀, lanes 5–8) rats treated (+) or not (–) with PTU or T₄. One Northern blot was sequentially hybridized with PS, S1, S3 and 18 S (upper panels); a second blot was hybridized with S, P1, K1 and 18 S (lower panels). Autoradiography was with Fuji RX film for 1 day (18 S), 3 days (S1), 4 days (S2, S3, K1) or 8 days (P1) or with Agfa RPC film for 1 day (PS).

were unchanged in the male rat kidney (Fig. 2). However, PS mRNA levels in the female kidney appear to be lowered 50 % by PTU administration, essentially unchanged by T₄ and restored to normal levels by the administration of T₄ to PTU-treated female rats. Although changes in renal K1 mRNA levels in both male and female rats were more variable, a similar pattern to that of PS was observed. K1 mRNA levels in the male kidney were not consistently markedly affected, and PTU treatment tended to lower (20–40 %) K1 mRNA levels in the female kidney (Fig. 2). The hybridization pattern obtained with gene-specific probes for S3 and P1, the two kallikrein gene family members known to be expressed in the rat prostate, is shown in Fig. 3 (lanes 5–8).

Fig. 2. Hybridization of ³²P-labelled PS- and K1-kallikrein-gene-specific oligonucleotide probes with 25 µg of kidney total RNA from male (♂) and female (♀) rats treated (+) or not (–) with PTU, T₄ or PTU/T₄. One Northern blot was sequentially hybridized with PS and 18 S; a second blot was hybridized with K1 and 18 S. Autoradiography was with Fuji RX film for 1 day (PS, 18 S) or 4 days (K1).

For comparison, and as an internal control, we included SMG RNA from the same experimental groups (lanes 1–4) on the same Northern blot. In contrast with the SMG, where the magnitude of changes for S3 and P1 mRNA levels is dramatic and consistent with that observed above (Fig. 1), the changes, if any, in prostatic S3/P1 mRNA levels relative to 18 S-RNA levels were less marked and much more variable with manipulation of thyroid-hormone status. In addition, we have detected a novel mRNA species with an exon-3-specific (Fig. 3, right panels), but not exon-2-specific (results not shown), P1 gene probe in the rat testis, an observation which was also recently made in a concurrent, but independent, study by Brady *et al.* [10]. This testicular mRNA species was only

Fig. 3. Hybridization of ³²P-labelled S3- and P1-kallikrein-gene-specific oligonucleotide probes and rat 18 S rRNA control probe with 25 µg of total RNA from SMG (lanes 1-4), prostate (lanes 5-8) and testis (lanes 9-12) of male rats left untreated (lanes 1, 5 and 9) or treated with PTU (lanes 2, 6 and 10), T₄ (lanes 3, 7 and 11) and PTU/T₄ (lanes 4, 8 and 12)

One Northern blot was sequentially hybridized with S3 and 18 S RNA (upper panels); a second blot was hybridized with P1 and 18 S (lower panels). Autoradiography was with Kodak XAR-5 film for 1 day (P1) or with Fuji RX film for 7 days (S3) or 8 days (18 S).

apparent under less stringent hybridization conditions (30 °C instead of 37 °C), although the wash conditions were relatively stringent (0.1 × SSC at 30 °C). We interpret this as evidence for the expression in the testis of an additional as yet uncharacterized kallikrein gene family member, similar in sequence to, but not identical with, P1 over the region of the exon-3-specific probe, and dissimilar over the exon-2 region. The levels of this testicular P1-like mRNA were essentially unchanged by thyroid hormone.

Finally, we have examined the effect of thyroid-hormonal manipulation on AP PS mRNA levels in both the male and female rat (Fig. 4). The expected pattern of PS gene expression in the rat pituitary was observed (female > male); female AP PS mRNA levels were unchanged with thyroid-hormone treatment, and male levels were too low to detect reliable changes. The blot was rehybridized with a cDNA probe for prolactin, given the previous demonstration of congruent changes in AP kallikrein (PS) and prolactin mRNA levels on manipulation of oestrogen status [13,15]. As expected, female AP prolactin mRNA levels were higher than those of the male, and the AP prolactin mRNA levels in both sexes were altered to various extents by thyroid-hormone treatment.

Fig. 4. Hybridization of ³²P-labelled PS-kallikrein-gene-specific oligonucleotide probe, prolactin cDNA or rat 18 S rRNA control probe with 25 µg of total RNA from control SMG (lane 1), male (♂) AP (lanes 2-5) and female (♀) AP (lanes 6-9) from control (lanes 2 and 6), PTU (lanes 3 and 7), T₄ (lanes 4 and 8) and PTU/T₄ (lanes 5 and 9)-treated animals

Autoradiography was with Fuji RX film for 4 days (PS) or with Agfa RP1 film for 1 day (PRL, 18 S).

DISCUSSION

We have demonstrated that the expression of the rat kallikrein gene family members (PS, S1, S2, S3, K1 and P1) in the rat SMG, kidney, prostate and AP is differentially affected by thyroid-hormone status. The expression of five of the rat kallikrein gene family members (S1, S2, S3, K1, P1) so far studied in the SMG has now been shown to be regulated by changes in thyroid-hormone status, in addition to their previously described androgen-dependence [14]. These changes presumably reflect the previously reported changes in kallikrein immunoreactivity, enzyme activity or mRNA levels as detected with a tissue kallikrein cDNA probe in the rat SMG on testosterone or T₄ administration [19,20]. In contrast, but in keeping with the previously reported androgen-independence of PS gene expression in this tissue, true kallikrein (PS) mRNA levels in the SMG were not altered by changes in thyroid-hormone status. The lack of effect observed here for true-kallikrein-mRNA levels

in either the male or female SMG is in keeping with that reported previously for mGK6 (renal kallikrein) in the female mouse SMG after T_4 administration [21]. Indeed, it would seem that true/renal kallikrein is the one family member, so far studied, for which expression in either the mouse or rat SMG does not appear to be androgen- and/or thyroid-hormone-dependent [14,21,71].

In contrast with those of the SMG, renal mRNA levels of both K1 and PS were essentially unchanged in these studies by thyroid status, and in previous studies [14] by androgen status. Consistent with these findings, renal kallikrein enzyme activity and immunoreactivity have been shown to be unaffected by thyroid-hormone administration to intact female or castrated male rats, except for an unexplained decrease in immunoreactive levels in the T_4 -treated castrate male rat [19]. Similarly, S3 and, to a lesser extent, P1 gene expression, which have been previously shown to be highly androgen-dependent in both the prostate and SMG [14], were also essentially unchanged in the prostate by thyroid-hormone status. The latter observation, in particular, underscores the tissue-specificity of the hormonal regulation of this gene family.

Similarly, changes in thyroid-hormonal status did not affect PS mRNA levels in the female AP. The expression of this kallikrein gene in the AP has been previously shown to be oestrogen-dependent in a manner similar to that of prolactin [12,13,15–18]. In contrast, in the present study, AP prolactin mRNA levels, particularly in the male, would appear to be thyroid-hormone-responsive. Thus these data provide the first demonstration that kallikrein and prolactin mRNA levels are not always co-ordinately regulated, as has been previously reported [12,13,15].

The mechanism of differential thyroid-hormone regulation of kallikrein genes in different tissues is poorly understood. Whether this reflects the presence of thyroid-hormone receptor responsive elements in the 5' promoter regions of some kallikrein genes (S1, S2, S3, P1 and K1), but not PS, awaits further characterization of the regulatory regions of these genes. However, in a recent study of T_3 -receptor binding to 5' flanking DNA of two mouse kallikrein genes, the more thyroid-hormone-responsive gene mGK3 did not exhibit T_3 -receptor binding [28]. In contrast, mGK6, for which cellular (detected by hybridization *in situ*), but not total, tissue mRNA levels appear to be down-regulated by T_4 [21], showed T_3 -receptor binding to multiple regions of its 5'-flanking DNA [28]. The physiological significance of this finding, however, is as yet unclear.

It is also possible that the hormone response may be secondary to other cellular events, such as cytodifferentiation or proliferation, as has been shown for the expression of some kallikrein gene family members in response to androgen or thyroid-hormone treatment in the mouse SMG [21]. However, renin mRNA levels in the mouse SMG have also been shown to be thyroid-hormone-responsive, with a rapid increase within 5 h of T_3 administration [29]. Such rapid effects are consistent with a direct transcriptional activation of renin gene expression and/or mRNA stabilization. Although a combination of the above may explain the thyroid-hormone-responsiveness of S1, S2, S3, K1, P1, but not PS, in the rat SMG, other mechanisms must also be involved, given the relative lack of responsiveness of the expression of these kallikrein gene family members in other tissues (kidney, AP), where thyroid-hormone responses have been clearly demonstrated in other systems [30–34]. Moreover, these responses may also be mediated by different members of the family of thyroid-hormone receptors which appear to be selectively expressed in different tissues [35–37].

In addition, we have demonstrated the expression of a novel P1-like kallikrein gene, which is not regulated by thyroid

hormone, in the rat testis. From differential hybridization studies it is not P1 itself being expressed, but a gene similar in sequence to, but not identical with, exon 3 of P1, as has been recently noted by Brady *et al.* [10] in an independent study. Presumably this constitutes another, as yet uncharacterized, member of the rat kallikrein gene family. Recently, in two genomic cloning studies, ten members of the rat kallikrein gene family were cloned and fully/partially sequenced (rGK-1–rGK-8 [7]; RSKG-7 and RSKG-3 [6]). Of these, rGK-1 encodes true kallikrein (PS), rGK-2 encodes tonin (S2) and rGK-8 has been shown to encode P1. Thus a conservative estimate of the size of the rat kallikrein gene family must be at least 11 members, namely rGK-1 (PS), rGK-2 (S2), rGK-8 (P1), rGK-3–rGK-7, RSKG-7, RSKG-8 and a previously reported pseudogene, RSKG-9 [5]. Whether one of these newly characterized genes will encode other previously described mRNAs (S1, S3, K1) and/or this novel P1-like mRNA expressed in the rat testis is yet to be determined.

In summary, the demonstration of the tissue-specific thyroid-hormone-responsiveness of the expression of various kallikrein gene family members (S1, S2, S3, P1, K1) in the rat SMG compared with the rat kidney, prostate, testis and AP, and the demonstration of a potential new family member being expressed in the rat testis, has extended the concept of tissue-specific hormonal regulation and expression of the rat kallikrein gene family. The further delineation of tissue-specific patterns of expression/hormonal regulation for the recently characterized new kallikrein gene family members [6,7] should help to identify tissue-specific modifiers/regulators and elucidate the potential physiological and/or functional roles of gene family members in these tissues.

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