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## Kallikrein-like prorenin-converting enzymes in inbred hypertensive mice

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### Abstract

Kallikreins are a group of serine proteases and are distinguished by having serine residue at their active site. Their general function is to convert inactive pro-peptide into its biologically active form. In recent years, emerging evidence indicates that some kallikrein–kinin enzymes also play a role in the modulation of renin–angiotensin system. These kallikrein-like prorenin converting enzymes act on renin–angiotensin by converting prorenin into biologically active renin. In this investigation, kallikrein-like prorenin converting enzyme (PRCE C) (mK9) is isolated from genetically inbred high blood pressure (BPH) and their normal counterparts (BPN) mice, and its protein levels are quantitated. Levels of mRNA expression are also compared. Additionally, localization of the enzyme is visualized by in situ hybridization histochemistry. Results indicated higher levels of PRCE C (mK9) enzyme in BPH mice in comparison to their normal counterparts. mRNA expression was also higher in BPH mice. In situ hybridization histochemistry results localized PRCE C (mK9) in the striated duct cells of submandibular gland.

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Normal blood pressure is a consequence of a delicate balance between blood pressure lowering kallikrein–kinin and blood pressure raising renin–angiotensin systems. Although other opposing systems also exist, they are not as intimately associated as these two systems. For example, angiotensin converting enzyme (ACE), on one hand, converts angiotensin I into biologically active angiotensin II, whereas, on the other hand, ACE also degrades biologically active bradykinin into inactive products. An imbalance of the two systems would result in high blood pressure.

Emerging evidence of past 10 years indicates that some mouse kallikrein-like enzymes behave like true (tissue/glandular) kallikreins [1]. Additionally, some of the kallikrein-like enzymes are also involved in processing the renin that releases angiotensin I from its substrate [2–7]. Three kallikrein-like enzymes that convert inactive prorenin to biologically active renin are mK9, mK13, and mK22 [8,9]. These three kallikrein-like enzymes, because of their effect on renin, are also des-

ignated as prorenin converting enzymes (PRCE) C, B, and A, respectively. They were first discovered as proteins bound to epidermal growth factors and were called epidermal growth factor-binding proteins (EGF-BP) C, B, and A [10–12].

Schlager [13–15], by eight-way cross of mice strains exhibiting high blood pressure and then by selective inbreeding for 50 generations, has developed a high blood pressure (BPH) line. Mean systolic blood pressure of BPH adult mice is 140 mmHg. The normal blood pressure (BPN) line derived from high blood pressure ancestors has mean systolic blood pressure of 97 mm Hg [16]. Thus, the BPH mouse model is suitable for evaluating individual enzyme of both kallikrein–kinin and renin–angiotensin systems. Additionally, because BPH mouse line is derived from the ancestors of BPN line, there is a considerable reduction of genetic variability between BPH and BPN strains.

In a previous study [17], we have isolated and identified PRCE C (mK9). In the present investigation, we wanted to know whether levels of kallikrein-like enzyme PRCE C (mK9) and its mRNA are altered in mice that are genetically inbred for high blood pressure.

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## Materials and methods

**Animals.** Two months old male mice genetically inbred for high blood pressure (BPH) and their normal counterparts (BPN) were purchased from Jackson Laboratories, Bar Harbor, Maine, and kept under normal conditions at animal care facility for additional three months. At the end of five months, the animals were sacrificed under deep anaesthesia and their submandibular glands were surgically removed.

**Tissue preparation.** Part of the surgically removed submandibular gland was frozen in liquid nitrogen for further use, whereas the rest of it was fixed in chilled phosphate buffered saline (PBS) containing 3% paraformaldehyde and 0.02% diethylpyrocarbonate for 2–3 h.

**Preparation of tissue extract.** One hundred milligrams of frozen submandibular gland was homogenized in 500  $\mu$ l of distilled water in a Douncer on ice. Homogenized glands were placed in an Eppendorf tube and centrifuged at 14,000 rpm for 10 min. The supernatant was aliquoted and stored at  $-70^{\circ}\text{C}$ .

**Isolation of protein by HPLC.** Known amount of aqueous extracted tissue was dissolved in equal amount of 0.1% TFA and centrifuged to remove insoluble material. The protein was subjected to HPLC on a Delta Pak-C18 300A column using 0.1% TFA and a gradient of acetonitrile containing 0.1% TFA at 1 ml/min. The peak fraction containing the protein of interest was immediately lyophilized.

**Protein sequencing.** Fractions representing major peaks were subjected to protein sequencing. The protein was attached to functionalized 1,4-diphenylene diisothiocyanate (DITC) and arylamine (AA) membrane (Milligen/Millipore) and sequenced using a Milligen protein sequencer (Model 6600) with on-line HPLC for PTH-amino acid detection.

**Protein estimation.** Protein was estimated by Bradford method, using a Bio-Rad kit for microassay of proteins. Bovine serum albumin was used as standard.

**Total RNA isolation.** The RNA of submandibular gland was isolated by using Qiagen Midi RNA isolation Kit (Cat No. 75144). Purity of RNA was checked and the amount of RNA was estimated by spectrophotometer (Gene Quant Pro. Amersham Pharmacia Biotech). The purity of isolated RNA was also checked by the agarose–formaldehyde gel.

**Dot blots.** RNA (10, 1, and 0.1  $\mu$ g) RNA from BPH and BPN mice submandibular glands were blotted and then crosslinked to nitrocellulose membrane by ultraviolet light (UV). The membrane was then prehybridized at  $42^{\circ}\text{C}$  with prehybridization solution (4 ml  $2\times$  SSC, 5 ml 100% formamide, 1000  $\mu$ l  $50\times$  dextran, and 100  $\mu$ l (100  $\mu$ g/ml) denatured salmon sperm DNA). After 2–3 h of prehybridization, appropriate denatured probe was added to fresh prehybridization solution in a sealing bag. The bag was incubated at  $42^{\circ}\text{C}$  in a shaker water bath for 16–20 h. The membrane was washed with  $2\times$  SSC several times for 2 h and was subjected to autoradiography at  $-70^{\circ}\text{C}$  for several hours.

**Northern blots.** Thirty micrograms of total RNA from BPH and BPN mice submandibular glands was dissolved in RNase free water and separated on 1% denatured agarose–formaldehyde gel, using MOPS buffer. The gel was then placed on nitrocellulose membrane and RNA was transferred (16–20 h). The transferred RNA was fixed by UV crosslinker. Nitrocellulose membrane was prehybridized at  $42^{\circ}\text{C}$  with hybridization solution. Rest of the method is described in Dot blots.

**In situ hybridization histochemistry.** BPH and BPN submandibular gland tissues were fixed in 3% paraformaldehyde. Following a wash with cold, the tissue was subjected to cryostat sectioning. Fixed tissue was placed on a block. Fifteen micrometers thick sections were cut simultaneously from submandibular glands of BPN and BPH mice. The sections were placed on glass slides coated with 2% 3-aminopropyltriethoxysilane. They were stored at  $-4^{\circ}\text{C}$  for 24 h. For in situ hybridization with oligonucleotide probes, sections were washed with

PBS and incubated with  $2\times$  SSC for 10 min. They were prehybridized for 1 h with prehybridization solution at room temperature [deionized formamide (5 ml), SSC( $20\times$ ) 2 ml, Denhardt's ( $50\times$ ) 0.2 ml, Herring sperm DNA (heat denatured, 10 mg/ml) 0.5 ml, Yeast tRNA (10 mg/ml) 0.25 ml, and Daxtran sulfate (50%) 2.0 ml]. Sections were then washed with  $2\times$  SSC, and incubated overnight at  $37^{\circ}\text{C}$  with digoxigenin labeled oligonucleotides prepared in the prehybridization solution at a concentration of approximately 280 ng of oligonucleotide/ml of prehybridization solution. Slides containing sections were washed first with  $2\times$  SSC for 1 h and then with  $1\times$  SSC for 1 h.

For immunological detection, slides were washed in buffer #1 (100 mM Tris–HCl, 150 mM NaCl; pH 7.5) and incubated in the same buffer containing 2% normal sheep serum and 0.3% Triton X-100 for 30 minutes. Three hundred microliters of diluted anti-digoxigenin conjugate (Boehringer–Mannheim) was applied onto the slides and incubated in a humidified chamber for 2–4 h. After washing the slides with buffer #1 for 10 min and with buffer #2 (100 mM Tris–HCl, 100 mM NaCl, 50 mM MgCl<sub>2</sub>; pH 9.5) for 10 min, color solution was applied to the slides. Staining solution was made by adding 45  $\mu$ l NBT, 35  $\mu$ l X-phosphate (Boehringer–Mannheim), and 2.4 mg Levamisole to 10 ml of buffer #2. Slides were monitored for optimal color development in the absence of light. The color reaction was stopped by washing slides in buffer #3 (10 mM Tris–HCl, 1 mM EDTA; pH 8.0). They were dehydrated in graded series of alcohol, cleared in xylene, and mounted in permount medium.

**Oligonucleotide synthesis and labeling.** For in situ hybridization a 24-mer oligonucleotide probe corresponding to a variable region of exon 4 of mK9 specific mRNA was commercially synthesized (Life Technologies). The probe sequence is 5'-GTCCAAAAATGCAAAAGATCTCCAG-3'. Thirty-five picomoles of oligonucleotide was labeled with digoxigenin-11-dUTP, using DNA tailing kit (Boehringer–Mannheim). For dot and Northern blot analysis, the probe was labeled using [ $\gamma$ -<sup>32</sup>P]ATP in the presence of T4 polynucleotide kinase (Promega).

**Densitometric analysis.** Spot densitometric analysis was carried out using MultiImage light cabinet filter system attached to AlphaImager 2000 documentation and analysis system. Results of the analysis were corrected for background activity and expressed as integrated density value (IDV), %, and average (IDV/area).

## Results and discussion

Fig. 1A shows the result of reverse-phase HPLC after loading 60  $\mu$ g of soluble protein from submandibular gland of BPH mice. In this figure the amount of the protein in peak fraction #4, as judged by the height and the width of the peak, is substantially higher in comparison to BPN mice.

Fig. 1B shows result of reverse-phase HPLC after loading 60  $\mu$ g of soluble protein from the submandibular gland of BPN mice. Many peaks are visible.

Fractions of major peaks were collected, pooled, lyophilized, and subjected to amino acid analysis. The sequences obtained were then compared and matched with published or deduced protein sequences. In this figure peak #4 corresponds to PRCE C (mK9).

Fig. 2A shows quantitation of the peak #4 by integrating peak width and height of four runs. Stippled bar represents integrated area of peak #4 containing PRCE C (mK9) of BPH mice and dark bar shows PRCE C (mK9) from BPN mice.

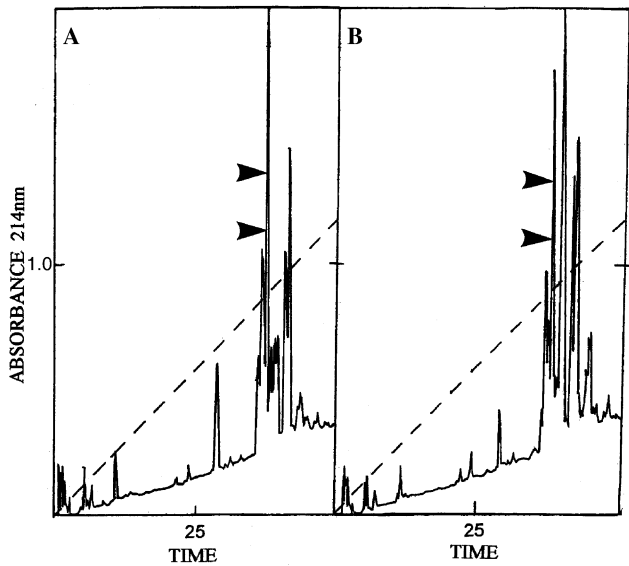


Fig. 1. (A) HPLC run of an aqueous extract from submandibular gland of high blood pressure (BPH) mice. Protein load is 60  $\mu$ g. Arrow shows the peak containing PRCE C (mK9). Note that the height and the width of BPH peak are greater than that of BPN peak. (B) HPLC run of an aqueous extract of normal blood pressure (BPN) mice submandibular gland using C18 column. In this run amount of protein loaded is 60  $\mu$ g. Arrow indicates the peak containing PRCE C (mK9).

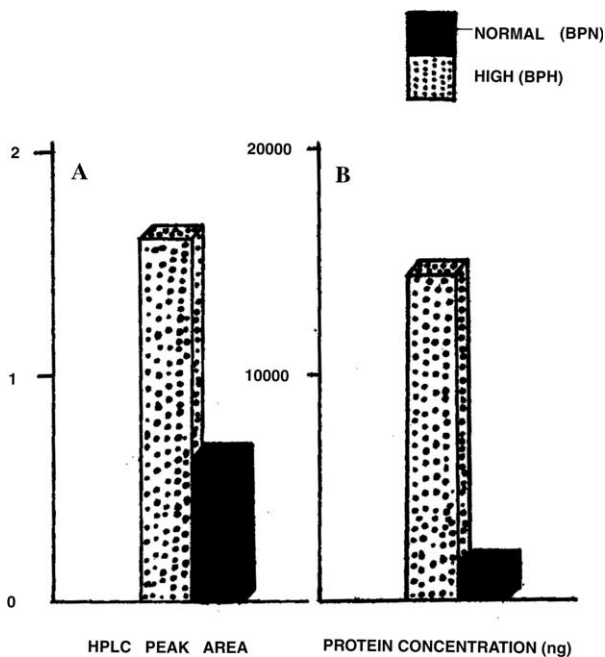
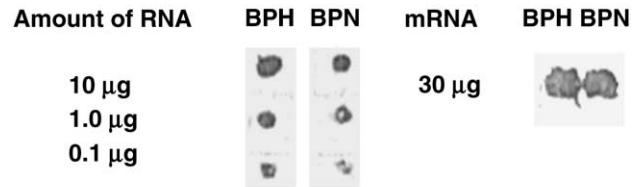


Fig. 2. (A) Quantitation of the peak areas by integrating the height and the width from Fig. 1. Stippled bar indicates the area of the peak from BPH mice gland, whereas dark bar shows the area of the peak from BPN gland. From this figure it is clear that in BPH mice PRCE C (mK9) amount is larger than that seen in BPN mice. (B) Protein estimations of fractions obtained from PRCE C (mK9) peaks of BPH and BPN mice. The amount of the protein expression in the peak fraction obtained from BPH mice gland is much greater than peak fraction obtained from BPN mice.



A Dot blot

B Northern blot

Fig. 3. (A) Dot blot analysis of RNA from BPH and BPN mice submandibular glands. Over the range of 10.0–0.01  $\mu$ g the concentration of PRCE C (mK9) specific RNA in BPH mice is higher than BPN mice. (B) Northern blot analysis of RNA from BPH and BPN mice submandibular glands. Expression of PRCE C (mK9) specific RNA in BPH mice is slightly higher than that seen in BPN mice.

Fig. 2B indicates protein estimation of peak #4 from lyophilized fractions of three glands containing PRCE C (mK9). It is obvious from the figure that protein concentration is substantially higher in BPH mice in comparison to BPN mice. Thus, it is obvious that in BPH mice there is a higher expression of PRCE C (mK9) enzyme in comparison to BPN mice.

Fig. 3 shows dot blot analysis of total RNA from BPN and BPH mice submandibular glands using 24-mer  $^{32}$ P labeled probe under high stringency conditions. It is clear from this figure that over the loading range of 10–0.1  $\mu$ g of total RNA, there is higher expression of PRCE C (mK9) specific mRNA in BPH mice submandibular gland.

Table 1 shows spot densitometric quantitation of isolated RNA. It is obvious from this table that PRCE C (mK9) expression is higher in BPH mice than seen in BPN mice and that these results concur with the results shown in Fig. 3.

Fig. 3B shows results obtained from Northern blot analysis of BPH and BPN mice submandibular glands RNA. There appears to be higher expression of PRCE C (mK9) in BPH mice.

Table 2 shows Northern blot spot densitometric analysis of BPH and BPN mice submandibular glands

Table 1  
Spot densitometry of mouse submandibular gland kallikrein-like PRCE C (mK9) mRNA

	Total RNA ( $\mu$ g)	Integrated density value	%	Average
BPH	10.0	30,008	49.6	62
	1.0	7260	12.0	15
	0.1	3388	5.6	7
BPN	10.0	16,940	28.0	35
	1.0	2420	4.0	5
	0.1	484	0.8	1

Integrated density value, sum of all the pixel values after background correction. %, the percentage that each circle contributed to the total density measured taking background into consideration. Average, the average value of the pixels enclosed after background correction.

Table 2  
Northern blot spot densitometry of the mouse submandibular gland prorenin converting enzyme C (kallikrein mK9)

	Loaded RNA ( $\mu\text{g}$ )	Integrated density value	%	Average
BPH	30	6384	24	153
BPN	30	5852	22	149

Integrated density value, sum of all the pixel values after background correction. %, the percentage that each circle contributed to the total density measured taking background into consideration. Average, the average value of the pixels enclosed after background correction.

PRCE C (mK9) specific mRNA. With a load of 30  $\mu\text{g}$ , the expression of PRCE C (mK9) in BPH mice is slightly higher than that of BPN mice. Thus, dot blot and Northern blot results and dot blot and Northern blot quantitations support the results obtained above from protein estimation and strengthen the view that there is higher expression of PRCE C (mK9) enzyme in BPH mice submandibular gland in comparison to their normal counterparts.

Fig. 4A shows result of in situ hybridization immunocytochemistry using digoxigenin labeled oligonucleotide for the detection of PRCE C (mK9) specific mRNA of BPN mice. The expression is confined to striated duct cells. Acinar and neck cells are not stained. Similarly, no staining was present in the connective tissue around larger ducts or blood vessels. More mRNA seems to be present around the nuclear and basal regions of these cells. However, the concentration of PRCE C (mK9) specific mRNA appears to vary from cell to cell.

Fig. 4B shows in situ hybridization immunocytochemistry, using digoxigenin labeled oligonucleotide, for the

detection of PRCE C (mK9) specific mRNA in BPH mice. PRCE C (mK9) specific mRNA, like BPN mice, is confined to striated duct cells only.

However, the expression of this mRNA appears to be much more than that seen in BPN mice submandibular gland. Also, the localization of mRNA in these cells appears to extend into the apical regions of these cells. All striated duct cells exhibited PRCE C (mK9). Our investigation is the first study to evaluate PRCE C (mK9) expression in BPH mice. From the results of protein estimations and RNA expression it is clear that PRCE C (mK9) is expressed at higher level in BPH mice. However, the exact mechanism of high PRCE C (mK9) at the molecular level is not clear at all and requires further investigation. We are currently analyzing the promoter region of PRCE C (mK9) of BPH and BPN strains of mice for its sequence and DNA binding proteins.

The increased expression of PRCE C (mK9) in BPH mice raises the possibility of its involvement in the genesis of essential hypertension. It has been shown that in guinea pig, mK9 is induced by thyronine [18]. In another study [19] it has been shown that in rats, thyronine induces hypertension via renin angiotensin system. Therefore, it is likely that in these rats thyroxine induced hypertension may be due to increased activity of mK9. A similar mechanism of action could be speculated in mice genetically that are selected for high blood pressure and used in the present study. Additional studies, however, are needed to verify thyroxine induced expression of PRCE C (mK9) in BPH strain of mice. In summary, in BPH mice submandibular gland PRCE C (mK9) enzyme is elevated and expression of PRCE C (mK9) specific RNA is also higher.

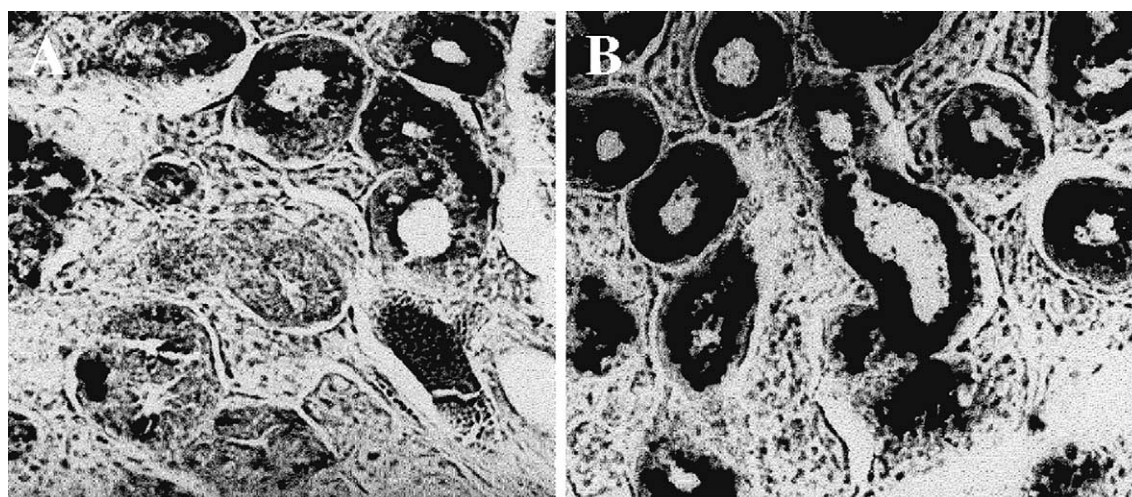


Fig. 4. In situ hybridization histochemistry of mice submandibular gland for PRCE C (mK9) specific RNA. (A) Fifteen micrometers thick section of BPN mice submandibular gland showing striated ducts with a distinct lumen. PRCE C (mK9) is located in the striated duct cells (darker appearing cells). However, not all striated duct cells appear darker. (B) Fifteen micrometers thick sections of BPH mice submandibular gland. All striated duct cells are darker in appearance, suggesting higher expression of PRCE C (mK9) in BPH mice. No staining was observed in the acinar cells or connective tissue of BPH or BPN mice.

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