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# Induction of pro-renin converting enzyme mk9 by thyroid hormone in the guinea-pig liver

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**Abstract**

Kallikreins are a group of specific serine proteases and are an integral part of kallikrein–kinin system. The kallikrein–kinin system is hypotensive in nature and counteracts with the renin–angiotensin system in the maintenance of normal blood pressure. So far, four kallikrein-like enzymes, namely, mK9, mK13, mK22, and mK26, have been known to convert the inactive pro-renin into biologically active renin. Some of these enzymes are induced by the thyroid hormone. In the proposed study, we investigated the effects of thyroid hormone on the expression of genes for mk9, mk13, and mk22 enzymes. We used guinea pigs as models because these animals share many characteristics in common to humans. Male adult guinea pigs were intramuscularly injected with 2 mg/kg body weight of thyronine. Forty-eight hours following the last injection, the liver was processed for Northern blot analysis using labeled mK9, mK13, and mK22 specific RNA probes. Only mK9 was found to be transcriptionally regulated by the hormone. 

*Keywords:* Pro-renin converting enzyme; Kallikrein; mRNA; Guinea-pig liver; Thyronine; Cyclohexamide; Actinomycin D

Kallikreins and kallikrein-like enzymes are serine proteases whose major function is to convert the inactive pro-peptide (pro-hormone) into a biologically active peptide (hormone). True kallikrein (tissue/glandular) releases biologically active kinin (bradykinin) from the α-2 globulin fraction of the plasma and, thus, is an integral part of the kallikrein–kinin system which is hypotensive in nature [1]. However, in recent years evidence is accumulating which suggests that tissue kallikreins may also be involved in the modulation of the renin–angiotensin system [2,3]. This group of serine proteases converts inactive pro-renin to active renin which then releases angiotensin I from the substrate angiotensinogen.

A significant number of kallikrein genes have been identified in mammalian tissues. Twenty genes have been reported in rat whereas 25 genes have been reported in mice [4,5]. However, only a few of these genes have been characterized (6 in rats [6], 10 in mice [7], 4 in humans [8], 3 in hamsters [9], and 3 in guinea pigs [10]).

Hosoi et al. [11] have demonstrated the kinin generating properties of mouse kallikreins mK9, mK13, and mK22 by using low and high molecular kininogens as substrates. Their results indicated that all these three kallikreins release bradykinin significantly but at different rates. In a recent investigation Timm [12] determined the crystal structure of mouse kallikrein mK13 and found it to be similar to other members of the mammalian serine protease family.

Kim et al. [2] demonstrated that a mouse pro-renin converting enzyme cleaved mouse Ren-2 pro-renin to yield mature renin. Partial amino acid sequence analysis of purified pro-renin converting enzyme and nucleotide sequence analysis of its cDNA clone indicated it to be identical to mK13. Kim et al. [13,14] also demonstrated the presence of two pro-renin converting enzymes. One of the enzymes showed characteristics similar to those of mK13 whereas the other enzyme turned out to be mK9. In a recent investigation Kikkawa et al. [3] evaluated the pro-renin processing properties of mK9, mK13, and mK22 as well as those of true tissue/glandular

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kallikreins (mK1). Their results indicated that mK9, mK13, and mK22 converted mouse Ren-2 pro-renin to active renin whereas mK1 did not have any effect.

Some of the kallikrein enzymes are modulated by thyroid hormone receptors [15,16]. However, almost nothing is known in regard to the effects of thyroid hormone on the expression of the kallikrein genes. The objective of the present study was to investigate the effects of intramuscular injection of thyronine on the expression of mK9, mK13, and mK22 in guinea-pig liver. We used guinea pigs since it is not possible to conduct basic experiments on humans. Guinea pigs can effectively be used as animal models to study the kallikrein gene products for many reasons. A major reason is that the number of expressed kallikrein genes is few and their number is closer to humans, suggesting a more close and parallel function rather than a wide and diverse function as evidenced by a large number of expressed genes in rats or mice. Other reasons include histological similarity of placenta [17], certain common metabolic characteristics [18], and similarity in lung development [19]. In the present study, $^{32}$P-labeled oligonucleotide probes made against mK9, mK13, and mK22 mRNAs were used to detect the expression of these enzymes in the guinea-pig liver.

## Materials and methods

*Animals and treatment*. Twelve adult male guinea pigs (Hartley Strain) were obtained from Charles Laboratories and housed at the animal care facility, Meharry Medical College, Nashville, TN. They were divided into four groups of three each. Animals of group I were injected intramuscularly with two doses of 2 mg/kg of thyronine 24 h apart. Animals of the second group were simultaneously given intramuscularly two doses of 2 mg/kg of thyronine and 200 μg/kg of actinomycin D intraperitoneally. The third group of animals was given 2 mg/kg of thyronine intramuscularly and 200 μg of cyclohexamide intraperitoneally. Control group of animals was injected intramuscularly with 100 μl of 5 mM NaOH in normal saline. Forty-eight hours following the last injection all animals were sacrificed under deep anesthesia, their livers were taken out, and frozen immediately in liquid nitrogen for further use.

*RNA isolation*. Total RNA was isolated from 500 mg of rapidly frozen liver by using the Promega RNA isolation kit (cat # Z5110). Purity of the RNA preparation was checked and the amount of RNA was estimated by measuring the absorbance at 230, 260, and 280 nm. Ratios of values at 260 by 230 and 260 by 280 at greater than 1.6 and 1.7, respectively, were used as the criteria for establishing the purity of RNA.

*RNA probe synthesis, sequence, labeling, and hybridization*. Three 24-mer oligonucleotide probes corresponding to mouse mRNA of kallikreins mK9, mK13, and mK22 in the variable regions of exon 4 were synthesized. The sequences are:

5′-GTCCAAAATGCAAAAGATCTCCAG-3′ (mK9)

5′-TAAGACAGACCCTCTCTGGAATGA-3′ (mK13)

5′-GACCAAAACCCAAATGATCTCCAG-3′ (mK22)

The oligonucleotides were labeled with [$^{32}$P-γ]ATP in the presence of T4 polynucleotide kinase. Fifty picomole probe was incubated with 10 units of T4-kinase (Promega) and 150 μCi of [$^{32}$P-γ]ATP in kinase buffer. The reaction was terminated by heating at 56 °C for 10 min. The labeling was monitored by analyzing the reaction mixture with TLC. The labeled probes were further separated from free ATP by precipitation with alcohol [20]. Thirty μg of total RNA from different experiments was separated on 1% agarose containing formaldehyde and transferred onto nitrocellulose membrane. The membrane was hybridized with $^{32}$P-labeled probes for kallikreins and autoradiographed following a wash, first with low stringency solution (×2 SSC, 0.1% SDS at room temperature for 15 min) and then with a high stringency solution (×0.1 SSC, 0.1% SDS at 37 °C for 30 min).

*Densitometric analysis*. Spot densitometric analysis was carried out by using multiimage light cabinet filter system and Alphaimager 2000 documentation and analysis system. The density was expressed as integrated (IDV), % and average (IDV/area), taking into account the background correction.

## Results

### *Probe labeling*

Fig. 1 shows the $^{32}$P labeling of oligonucleotides by T4-kinase. It is clear that all three probes used in this experiment are labeled. The labeling was approximately 70%. The bottom part of this figure shows thin layer chromatography. Following high stringency wash there is 70% labeling of all three probes.

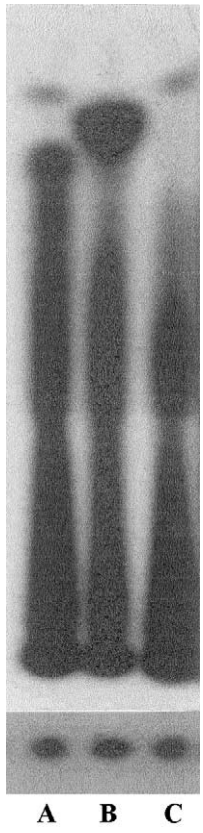


Fig. 1. Top part shows $^{32}$P labeling of oligonucleotide by T4-kinase. Seventy percent of the probe is labeled. The bottom part of this figure shows thin layer chromatography after high stringency wash. Approximately 70% of the probe is labeled: (A) probe mK9; (B) probe mK13; (C) probe mK22.

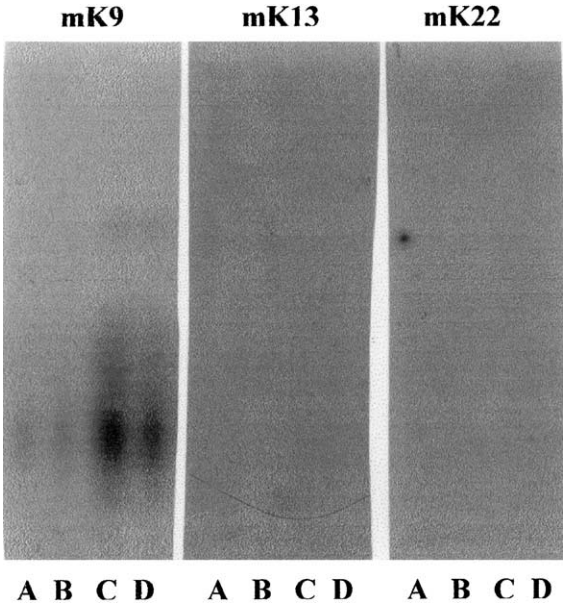


Fig. 2. (A) mK9, mK13, and mK22 specific mRNA expression in normal guinea-pig liver; (B) thyronine plus actinomycin D; (C) thyronine plus cyclohexamide; (D) thyronine only.

*Probe specific mRNA expression*

Our results indicate that, by using 24-mer $^{32}$P-labeled oligonucleotide made against mouse variable region of exon 4 mRNA, mK9 pro-renin converting enzyme specific mRNA is expressed in the liver of male adult guinea pigs at a lower concentration (Fig. 2A). However, no expression of mK13 or mK22 pro-renin converting enzyme specific mRNA has been detected. Since hybridization was carried out under high stringency conditions, it is unlikely that the binding of mK9 specific mRNA probe is unspecific.

*Effect of thyroid hormone*

Fig. 2D shows the effect of intramuscular injection of 2 mg/kg of thyronine on the expression of mK9 specific mRNA in the guinea-pig liver. The amount of mK9 specific mRNA expressed is substantially more than that expressed in a normal liver. However, there is no expression of mK13 or mK22 specific mRNA following intramuscular thyronine injection.

*Effect of thyroid hormone plus actinomycin D*

Fig. 2C shows the effect of thyronine (2 mg/kg) and actinomycin D (200 μg/kg) on the expression of mK9 specific mRNA in the guinea-pig liver. There is almost no detectable expression of mK9 specific mRNA. Similarly, mK13 or mK22 specific mRNA has not been detected.

*Effect of thyronine plus cyclohexamide*

Fig. 2B shows the effect of thyronine (2 mg/kg) and cyclohexamide (200 μg/kg) on the expression of mK9 specific mRNA in the guinea-pig liver. In this case there is substantially more expression of mK9 specific mRNA than that seen in the normal tissue or thyronine induced expression. However, mK13 or mK22 specific mRNA has not been detected at all.

Table 1 shows the densitometric analysis of Northern blots of guinea-pig mK9 specific mRNA. The highest expression is seen in guinea-pig liver treated with thyronine and cyclohexamide. This is followed by the liver treated with thyronine only. Normal liver shows a low expression of mK9 specific mRNA and actinomycin D almost completely blocks the expression. When translation is blocked by cyclohexamide, there is substantial accumulation of mK9 specific mRNA.

## Discussion

Results of the present investigation indicate that normal guinea-pig liver expresses mK9 specific mRNA. Although tissue kallikreins have been detected in other guinea-pig tissues [10,21] this is the first report on the presence of a specific family member of kallikrein which also possesses a pro-renin converting activity. Additionally, our results indicate that kallikreins mK13 and mK22 which are also pro-renin converting enzymes are not detected in the guinea-pig liver. Since mK13 and mK22 have only been found in NMRI, ICR, BPH, BPN, and BPL strains of mice, their non-detectability in the guinea-pig liver is not surprising. There are 25 distinct genes for kallikreins in mice whereas only three kallikrein genes are expressed in the guinea pig, sug-

Table 1
Spot densitometric analysis of guinea pig mK9 specific mRNA

| | Integrated density value (IDV) | Density (%) | Average (IDV/area) |
|---|---|---|---|
| Total mRNA: 30 μg | | | |
| Normal liver | 113,320 | 14.7 | 153 |
| Thyronine + actinomycin D | 112,480 | 14.7 | 152 |
| Thyronine + cyclohexamide | 280,800 | 36.6 | 200 |
| Thyronine only | 261,144 | 34.0 | 186 |

IDV, sum of all the pixel values after background correction; % Density is the percent that each ellipse contributes to the total density measured, taking the background into consideration; Average is the average value of the pixel enclosed after background correction.

gesting a more specific function of pro-renin converting enzyme in the guinea pig.

Thyroxine induces mK9 specific mRNA in the guinea-pig liver. This suggests a modulation by thyroid hormone of mK9. van Leeuwen et. al. [22] have shown that in mice some family members of kallikrein are also influenced by thyroid hormone or testosterone; however, in their study thyroid hormone's effect on mK9 has not been evaluated.

Thyroxine induction of mK9 specific mRNA can be blocked by simultaneous intraperitoneal administration of actinomycin D—an inhibitor of transcription. This suggests the binding of thyroxine–carrier protein complex to promotor upstream of the transcription initiation site. Barlow et al. [15] have demonstrated the presence of multiple binding sites for thyroid hormone receptor encoding kallikrein mK6 in mice.

Simultaneous thyroxine and cyclohexamide (an inhibitor of translation) administration resulted in the accumulation of mK9 specific mRNA indicating that thyroid hormone, in association with its carrier protein, directly affects the transcription of mK9 gene and not in an indirect fashion, by the induction of some other proteins.

Garcia et al. [16] have demonstrated that thyroxine treated rats showed increased mean arterial blood pressure and that this increase in blood pressure could be normalized by a simultaneous administration of Captopril-an ACE inhibitor and thyroxine. Our results indicate that thyroxine increases the mK9 specific mRNA in the guinea pig. Thus, the possibility is raised that thyroxine induced hypertension is mediated by the protein product kallikrein mK9 which is a pro-renin converting enzyme.

In summary, mRNA of kallikrein mK9-a pro-renin converting enzyme has been detected in the guinea-pig liver. This RNA is induced by thyroxine. The induction is blocked by actinomycin D but not by cyclohexamide. Possibility is raised that thyroxine induced hypertension is mediated by kallikrein mK9 in the guinea pig.

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