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CHARACTERIZATION OF ANGIOTENSIN II METABOLISM BY AMINOPEPTIDASE A

by

LIJUN SONG

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1996

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

CHARACTERIZATION OF ANGIOTENSIN II METABOLISM BY AMINOPEPTIDASE A

by

Lijun Song

Adviser: Professor Dennis P. Healy

The functions of the renin-angiotensin system (RAS) in regulating blood pressure and fluid volume have been known for a long time. The RAS has been implicated in the development of hypertension. Little is known about the regulation of angiotensin II (AII) metabolism. The first step in the metabolism of AII is by aminopeptidase A (APA) to hydrolyze the N-terminal aspartyl residue of AII to produce AIII, AIII is then degraded into inactive fragments by other peptidases. APA, a membrane-bound ectoenzyme, is generally believed to be an unregulated housekeeping enzyme. In order to understand the functional relationship between AII and APA in AII metabolism, my thesis is focused on the studies of APA localization and APA regulation by AII in rat tissues. In chapter 2, the localization of APA in rat peripheral tissues was studied. APA was localized to capillaries in posterior pituitary, sinusoids of adrenal cortex, and mesangial cells of kidney glomeruli. This localization is consistent with APA influencing blood pressure and fluid volume by affecting AII stimulated secretion of ADH from pituitary, synthesis

and release of aldosterone from the adrenal, and renal fluid homeostasis. In chapter 3, the localization of APA in cerebral microvessels was studied, APA was localized to pericytes, suggesting that APA may act as a part of the blood brain barrier and may play an important role in regulating the effects of AII on the brain. In chapter 4, the effect of AII on APA expression in the kidney of chronically AII-infused rats was studied. APA was elevated in glomeruli. In chapter 5, the regulation of APA by AII was studied in two-kidney one clip (2K-1C) Goldblatt hypertensive rats. APA was elevated in glomeruli from both clipped and unclipped kidneys. These results suggest that APA expression is regulated by AII. This is the first example of a bioactive peptide regulating the activity of its degrading peptidase in the peripheral tissues. Furthermore, these results suggest that stimulation of APA activity by AII may be a protective mechanism to minimize the adverse effects of AII in human hypertensive diseases.

DEDICATION

This thesis is dedicated to
my husband Hao Song and my son Rainbow Song;
my father and my mother.

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Chapter 1

INTRODUCTION

1. REVIEW 0F THE BIOLOGICAL SIGNIFICANCE OF ANGIOTENSIN II

The renin-angiotensin-system (RAS) is an important endocrine system for regulation of blood pressure and blood volume (Garrison and Peach, 1990). Angiotensinogen, the precursor protein of the RAS, is cleaved into angiotensin I (AI) in the circulation by renin, the rate-limiting enzyme. Renin is released from kidney juxtaglomerular cells in response to a variety of factors, including low perfusion pressure, sympathetic nerve stimulation, and low sodium load of early distal tubule via the macula densa (Garrison and Peach, 1990). Angiotensin converting enzyme (ACE) converts AI into the biologically active peptide angiotensin II (AII). AII exerts its effects by acting on angiotensin receptors that are coupled to the activation of phospholipase C or the inhibition of adenylyl cyclase via G-proteins (Guillemette et al., 1986; Peach, 1986). The biological effects of circulating AII include: 1. Direct stimulation and contraction of vascular smooth muscle cells (Dzau, 1987; Schelling et al., 1991). 2. Increasing the central sympathetic outflow; releasing catecholamines from adrenal medulla and sympathetic nerves; and amplifying postsynaptic response to norepinephrine (Fitzsimons, 1980; Peach, 1986). 3. Stimulation of the adrenal cortex to increase the synthesis and release of aldosterone (Bell et al., 1984). 4. Increase drinking by acting on brain circumventricular organs (Fitzsimons, 1980). 5. Reduction in the rate of renal blood flow and glomerular filtration, and increase reabsorption of

Na^+ in the lumen of proximal tubules by stimulating Na^+/H^+ exchange (Campese and Hsueh, 1983; Menard et al., 1984). The overall response to AII is an elevation of systemic blood pressure and blood volume.

AIII, (des-Asp^1)AII, has the same biological effects as AII, such as release of aldosterone from the adrenal (Blair-West et al., 1971), but it is generally less potent than AII (Goodfriend et al., 1977). However AIII is as effective as AII in stimulating drinking and increasing blood pressure in the brain (Wright et al., 1985; Wright and Harding, 1992).

Increasing evidence indicates that local RASs exist in many tissues and organs, especially in cardiovascular related structures of the brain, kidney, and arterial wall. Although renin is primarily released from the kidney (Garrison and Peach, 1990), it is also present in both endothelial cells and vascular smooth muscle cells (Okamura et al., 1992; Lilly et al., 1985). Angiotensinogen mRNA is present in the brain and vascular tissues (Campbell, 1987). High concentrations of ACE are found in the lung (Ryan, 1982), kidney and brain (Stier, 1989). AII and AII receptors are concentrated in many areas of brain (Unger et al., 1988; Moffett et al., 1987; Mendelsohn, 1984). AII receptors are also localized in different parts of the kidney, the anterior pituitary, cardiomyocytes, and sinusoids of the adrenal cortex (Mendelsohn, 1985). Two AII receptor subtypes are known. Both are G-protein coupled receptors with differences in both structure and function; the AT_1 receptor is a G-protein coupled receptor linked to the stimulation of phospholipase C (Sumners et al., 1991), while the AT_2 receptor is linked to the inhibition of phosphotyrosine phosphatase (Kambayashi et al., 1993).

Chronic hypertension is associated with arterial wall hypertrophy, increased collagen content and a reduction in arterial compliance (Hajdu et al., 1991). AII, either released from the arterial wall or from the circulation, can act as a trophic factor in stimulating proliferation and collagen synthesis of vascular smooth muscle cells in culture (Kato et al., 1991), while ACE inhibitors can reduce the medial thickness of cerebral vessels in spontaneously hypertensive rats (Clozel et al., 1989). AII thus exerts its effects on both the structure and tone of vasculature.

The beneficial effects of ACE inhibitors in the treatment of human hypertensive diseases have been known for a long time (Antonaccio, 1982; Williams, 1988). By inhibiting ACE to block the synthesis of AII (Garrison and Peach, 1990; Rocha et al., 1949), ACE inhibitors are effective for the treatment of human hypertension, especially renovascular hypertension (Williams, 1988; Garavaglia et al., 1988). ACE inhibitors are also effective in the treatment of congestive heart disease (Pfeffer et al., 1988).

Therefore, it is possible that hypertension could be caused by both endocrine and paracrine effects of AII on a variety of tissues, especially on the brain, kidney and blood vessels.

2. AII METABOLISM

A. Synthesis of angiotensin

Renin is the rate-limiting enzyme in the synthesis of angiotensin (Fig.1). Renin is an aspartyl protease which specifically hydrolyses the N-terminal Leu^{10}-Val^{11} bond of angiotensinogen, an alpha-2-globulin produced by the liver. The generated decapeptide AI is a substrate for ACE. ACE, a zinc dependent peptidyl dipeptidase, converts AI into the biologically active octapeptide AII. Biological and pharmacological studies of renin and ACE on RAS related hypertension have been reviewed extensively (Cushman et al., 1977; Deforrest et al., 1989; Haber, 1984; Johns and Ayers, 1984; Skeggs, 1984; Antonaccio, 1982; Garrison and Peach, 1990; Pinto et al., 1995). Pharmacological agents that block AII synthesis or AII receptors reduce blood pressure in both experimental animals and humans (Williams, 1988; Wong et al., 1990). Likewise the beneficial effects of ACE inhibitors on the treatment of human hypertensive diseases including congestive heart failure as a result of hypertension, myocardial infarction, essential hypertension and stroke are well known (Garg et al., 1995; Pfeffer et al., 1988; Lancaster et al., 1988; Garavaglia et al., 1988).

B. Degradation of angiotensin

Many enzymes, so called angiotensinases, are thought to be involved in the degradation of AII into inactive peptide fragments (Garrison and Peach, 1990). Studies on rat plasma AII indicate that AII is metabolized by a plasma enzyme, aminopeptidase A (glutamyl aminopeptidase, E.C.3.4.11.7), which hydrolyses the N-terminal aspartyl residue of AII to produce the heptapeptide AIII. AIII has pressor activity less than half that of AII in the peripheral tissues (Ahmand and Ward, 1990; Campbell et al., 1974; Nagatsu et al., 1965). Studies on rat brain RAS by Abhold and Harding (1988) further

demonstrate that AII is specifically converted into AIII by APA which cleaves the Asp^1-Arg^2 bond of AII (Fig.2). AIII is then sequentially degraded by other angiotensinases including aminopeptidase M (APM) cleaving the Arg^2-Val^3 bond of AIII to produce ANG(3-8). Ang (3-8), or so called AIV, has been shown to bind to distinct sites in the cerebral cortex and hippocampus and to facilitate memory process and regulate blood flow in the brain (Wright at al., 1995), and to stimulate aldosterone secretion from adrenal cortex (Blair-West et al., 1971). Prolyl endopeptidase cleaves the Pro^7-Phe^8 bond of AIII and dipeptidyl aminopeptidase III (DPPIII) cleaves the Val^3-Tyr^4 bond of ANG (3-8) (Abhold and Harding, 1988; Garrison and Peach, 1990).

APA is a membrane bound enzyme which cleaves specifically the N-terminal alpha-L-Glu and alpha-L-Asp residues from oligopeptides, including the N-terminal Asp residue from AI or AII and cholecystokinin-8. Recent studies on the amino acid sequences of both the murine B-lymphocyte differentiation antigen BP-1/6C3 and the human kidney differentiation antigen gp160 indicate that they had characteristics of APA. The amino acid sequence of the BP-1/6C3 antigen indicated that it shared homology with other zinc dependent metalloproteases. The BP-1/6C3 antigen also exhibited APA activity, was enhanced by Ca^{++} and may blocked by BP-1 antibody or amastatin, a inhibitor of APA (Wu et al., 1991). The cDNA of gp160 antigen from the epithelial cells of glomerulus and proximal tubule cells of the human kidney was shown to have 78% homology with the murine BP-1/6C3 antigen, and also had APA enzyme activity (Nanus et al., 1993). Aminopeptidase M (APM, E.C.3.4.11.2), also a membrane bound enzyme, cleaves all N-terminal L-amino acids residues from peptides or polypeptides including cleaving the N-terminal Arg residue of AIII to produce AIV.

Both APA and APM are glycoproteins and are inhibited by EDTA. Studies on the amino acid sequences of APM and APA (Wu et al., 1990; Wu et al., 1991) indicate that the two enzymes are 30% identical in their amino acid sequences. APA activity is Ca^{2+} and Zn^{2+} dependent but APM is only Zn^{2+} dependent (Kugler, 1982).

Angiotensin peptides are thought to be neurotransmitters or neuromodulators for the brain RAS (Saavedra, 1992; Wright and Harding, 1992). There are controversies about the identity of the active angiotensin peptide in the brain. Studies by Wright et al. (1985) suggest that intracerebroventricular (icv) injected AII and AIII are equipotent in stimulating drinking and evoking a pressor reaction. Several other studies indicate that AIII may be the active peptide in the brain (Moffet et al., 1987). Indeed, electrophysiological studies of angiotensin-stimulated changes in blood pressure (BP) and neuronal activity related to water drinking and BP in the brain suggest that AIII is more potent than AII is (Felix and Schlegel, 1978; Harding and Felix, 1987). Amastatin, an inhibitor of APA (Harding et al., 1987), significantly reduced the magnitude and duration of central pressor effects by icv injected AII. There is elevation in both magnitude and duration of central pressor effects of icv injected AIII in the presence of bestatin (Wright et al., 1990), an APM inhibitor blocking the degradation of AIII into smaller inactive fragments (Harding et al., 1987). Meanwhile, the central pressor effects of either AII or AIII icv injections are blocked by angiotensin receptor antagonist sarthran, suggesting that both peptides are acting on the same receptor in the brain (Wright et al., 1990). Interestingly, the $t_{1/2}$ of icv injected AII (23 sec.) is three times longer than that of AIII (7.7 sec.), while the $t_{1/2}$ of AII in plasma is similar to or shorter than AIII (Harding et al., 1986; Gaynes et al., 1978). Thus the rapid degradation of AIII

in the cerebrospinal fluid suggests that AIII may be more potent than previously considered (Wright et al., 1985; Wright and Harding, 1992). These results suggest that APA may be important in the central regulation of BP by converting AII to the active peptide AIII in the brain.

Studies by Bausback et al. (1988) on APA activity in porcine brain indicated that APA activity was concentrated more than ten fold in cerebral microvessels compared to the whole brain. APM activity was also concentrated in the cerebral microvessels from rat brain (Solhonne et al., 1987). Among all the bioactive peptides known to be associated with cerebral microvessels, AII is the only peptide containing an aspartyl or glutamyl residue in the N-terminus (Uddman and Edvinsson, 1989), suggesting that APA may be a highly selective peptidase for AII in the cerebral vasculature. The localization of APA immunoreactivity in the adventitium of cerebral microvessels (Healy and Wilk, 1993) is consistent with labeling of pericyte cells. Since the blood brain barrier is constituted by endothelial cells, smooth muscle cells and pericytes (Risau et al., 1990), APA associated with cerebral microvessels may play an important role in the function of the blood brain barrier by metabolizing circulating or locally produced AII.

Other angiotensin metabolites have also been shown to have activities in the brain. ANG(1-7), produced by prolyl-endopeptidase hydrolysis of the Pro^{7}-Phe^{8} bound of AI or AII (Fig.1), increases release of vasopressin from the neurohypophysis of rat brain (William et al., 1991; Schiavone et al., 1988). This fact suggests the possible existence of an alternative pathway for producing bioactive end products of angiotensin (ANG) in

the brain.

C. Regulation of angiotensin metabolism

The mechanism of feedback inhibition by AII on renin release is well known (Gibbons et al., 1984; Haber, 1986). The stimulation of the AII receptor is coupled to the activation of phospholipase C and hydrolysis of phospholipids, which result in the formation of arachidonic acid (Garrison and Peach, 1990; Antonipillai et al., 1989). 12-HETE, a product of 12-lipoxygenase from arachidonate following AII receptor stimulation, is believed to be responsible for the inhibition of renin release (Antonipillai et al., 1989).

The influence of AII on its degradative enzymes has not been demonstrated. Indeed, relatively little is known about peptide hormones regulating the activity of their degrading enzymes. One report from Vargas et al. (1994) indicates that pyroglutamyl peptidase II, an ectoenzyme of the metallopeptidase family that degrades thyrotropin-releasing hormone (Czekay and Bauer, 1993), was downregulated by TRH in the adenohypophyseal cells of the anterior pituitary. However, other peptidases, such as endopeptidase 24.15 for the degradation of luteinizing hormone releasing hormone (LHRH) released from the hypothalamus (Lasdun et al., 1990) and neutral endopeptidase 24.11 for the degradation of atrial natriuretic factor released from the atrium (Bralet et al., 1994; Seymour et al., 1991), are not known to be regulated by their substrate peptides.

In summary, it is possible that the "metabolism" of AII in cardiovascular-related tissues may either be a conversion step to smaller active fragments or a degradation step, depending on the tissue. Any factor that interferes with either the synthesis or metabolism of AII could influence the hypertensive effects of AII.

3. EXPERIMENTAL STUDIES ON THE FUNCTIONAL RELATIONSHIP BETWEEN AII AND APA

To study the role of aminopeptidase A in the metabolism of AII, my thesis is organized in the following order:

Chapter 2. DISTRIBUTION OF AMINOPEPTIDASE A IN PERIPHERAL TISSUES

Because of the endocrine and possible paracrine effects of AII in different tissues, the importance of APA in the metabolism of AII in different tissues of rat needs to be studied. Previous localization studies of APA in different tissues of rat have primarily utilized histochemical methods (Kugler, 1982; Lojda and Gossrau, 1980). There is a single immunohistochemical report using an antibody to BP-1/6C3 (Li et al., 1993). We have produced rabbit antiserum against APA (APAAb) purified from rat kidney in collaboration with Dr. Sherwin Wilk (Department of Pharmacology, Mount Sinai School of Medicine). We have also partially cloned rat kidney APA (Song et al, 1993). The cellular localization of APA in rat pituitary, lung, kidney, adrenal, liver and intestine was studied by immunocytochemistry with APAAb, and the labeling of APA

mRNA was carried out by in situ hybridization with APA ^{35}S-riboprobe synthesized from rat kidney APA cDNA. The cellular localization and APA enzyme activity in the kidney glomeruli were further studied by the immunofluorescent labeling and histochemical staining, respectively.

Chapter 3. CHARACTERIZATION OF AMINOPEPTIDASE A IN CEREBRAL MICROVESSELS

The brain contains all the components of RAS (Wright and Harding, 1992) and angiotensin peptides are involved in central regulation of BP. It has been suggested previously that APA may be a highly selective peptidase for the metabolism of AII in the brain. Previous studies on rat brain APA indicate that APA activity is enriched in cerebral microvessels (Bausback et al., 1988) and that APA immunostaining is primarily associated with the adventitium of cerebral microvessels, consistent with labeling of pericyte cells (Healy and Wilk, 1993). In order to confirm the localization of APA in the cerebral microvessels and understand the functional relationship between AII and APA in the brain, APA enzymatic assays and immunoblots were performed on isolated rat cerebral microvessels. The cellular localization of APA in rat brain was studied by immunocytochemistry with a alternative tissue fixation method and by in situ hybridization with an APA ^{35}S-riboprobe as mentioned above.

Chapter 4. EFFECT OF AII INFUSION ON KIDNEY APA ACTIVITY

Relatively little is known about the regulation of peptidases by their substrates. APA is believed to be an angiotensinase and an unregulated housekeeping enzyme (Garrison and Peach, 1990). However, Abhold and Harding (1988) suggest that APA is specifically involved in the first step of AII metabolism to AIII (Abhold and Harding, 1988). The first indication that AII might regulate APA activity came from a study by Wolf et al. (1990). Uninephrectomy and partial ablation of the contralateral kidney increased APA levels. This increase in APA levels was blocked by ACE inhibitor, suggesting that APA was upregulated by elevated levels of AII in this model. Based on the kidney having very high APA activity (Kugler, 1982), studies on APA regulation by AII in the kidney in response to AII infusion were conducted. The effects of AII on APA enzyme activity, immunoactivity and mRNA were measured.

Chapter 5. CHARACTERIZATION OF APA IN 2K-1C RENAL HYPERTENSIVE RATS

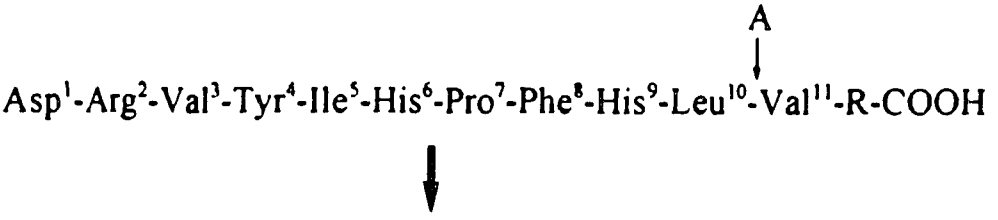
The studies of chronic AII infusion on APA activity in chapter 4 indicated that intrarenal APA was increased in the presence of augmented circulating and intrarenal AII. However, the possibility that glomerular hypertension as a result of AII influenced intrarenal APA expression could not be eliminated. By using the two-kidney one clip (2K-1C) Goldblatt hypertensive rat model, we were able to study effect of AII on kidney APA activity in absence of a hypertension. The methods that were utilized were the same as those used in chapter 4.

Chapter 6. DISCUSSION

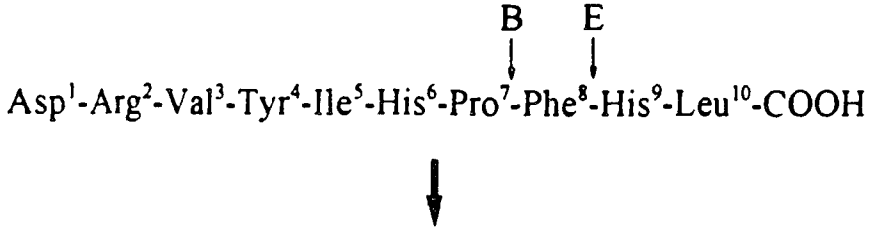
The results of the experiments reported here suggest that AII is able to regulate the activity of its principal degradative/converting enzyme APA. These results are discussed with regards to their implications on human cardiovascular/renal diseases.

General metabolism of the renin-angiotensin system

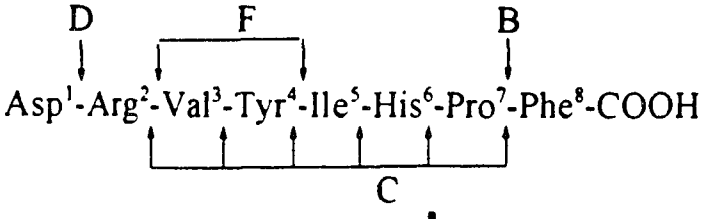
Angiotensinogen:



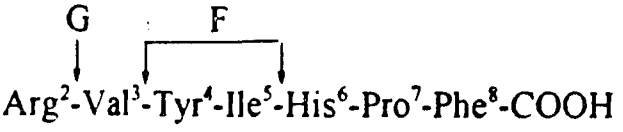
Angiotensin I:



Angiotensin II:



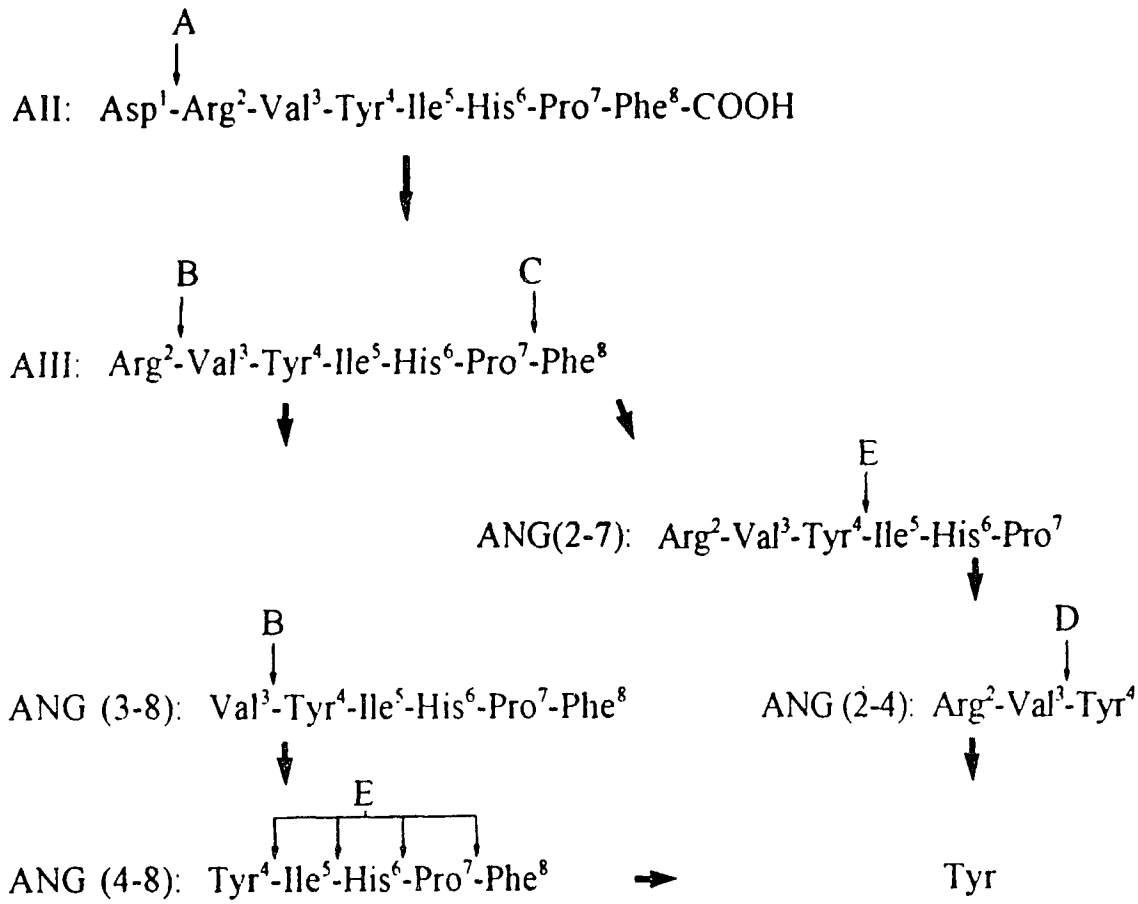
Angiotensin III:



A. Renin
B. Prolyl endopeptidase
C. Other angiotensinases
D. Aminopeptidase A
E. Angiotensin converting enzyme
F. Dipeptidyl aminopeptidase III
G. Aminopeptidase M

Figure 1

The metabolism of angiotensin II



A. Aminopeptidase A
B. Aminopeptidase M
C. Prolyl endopeptidase
D. Dipeptidyl aminopeptidase III
E. Other angiotensinases

ANG. Angiotensin
AII. Angiotensin II
AIII. Angiotensin III

Figure 2

Chapter 2

DISTRIBUTION OF AMINOPEPTIDASE A IN PERIPHERAL TISSUES

INTRODUCTION

The renin-angiotensin system (RAS) is involved in the regulation of blood pressure, body sodium and fluid homeostasis (Gross, 1968; Skeggs, 1984). The liver, endothelial cells and kidney juxtaglomerular cells are the major tissues for the production of angiotensinogen, ACE and renin, respectively (Campese and Hsueh, 1983; Ben-Ari and Garrison, 1988; Skeggs, 1984; Ryan, 1982). Recent biochemical and molecular studies indicate that local RASs exist in many tissues. Angiotensinogen and renin mRNA are present in many tissues, including the brain, pituitary, cardiomyocytes and endothelial cells of rat (Campbell, 1987; Healy et al., 1992; Okamura et al., 1992; Lilly et al., 1985; Moffett et al., 1987). AII receptors are localized in kidney mesangial cells, brush border of renal tubules, afferent and efferent arterioles of the kidney, adrenal cortex and medulla, plasma membranes of cardiomyocytes, anterior pituitary, and hepatocytes (Mendelsohn, 1985). Because of extrarenal production of AII and the existence of AII receptors on various tissues, it is possible that AII may exert its endocrine and/or paracrine effects on these tissues.

APA is a membrane-bound ectoenzyme that has been cloned from several species, including human and mouse (Nanus et al., 1993; Wu et al., 1990). A partial cDNA for rat kidney APA has been reported (Song et al., 1993). APA has been shown

to metabolize AII to AIII (Abhold and Harding, 1988). Biochemical and histochemical studies of APA in different tissues of rat indicate that the enzyme activity is highly enriched in the glomerulus and brush border of proximal tubule cells of kidney, and in the brush border of differentiated enterocytes and periapical parts of intestinal villi (Kugler, 1981; Kugler, 1982a; Lojda and Gossrau, 1980). APA has also been localized histochemically to many other tissues including the endothelial cells, vascular smooth muscle cells, pregnant uterus, hepatocyte membranes, and apical portion of duct cells in exocrine pancreas (Lojda and Gossrau, 1980). By using rabbit antiserum (APAAb) against APA purified from rat kidney and APA ^{35}S-riboprobe synthesized from APA cDNA of rat kidney, we have conducted immunocytochemical and in situ hybridization studies on the cellular localization of APA in rat pituitary, lung, kidney, adrenal, liver and intestine. These experiments indicate that APA has a broad tissue distribution in the rat and may be important in the regulation of the endocrine and paracrine effects of AII on the physiological functions of a variety of tissues.

MATERIALS/METHODS

Experimental animals

Male Sprague-Dawley rats (175-225 g, Charles River Breeding Laboratories, Wilmington, MA) were housed on a 12 h light/12 h dark schedule and allowed free access to food and water.

Isolation of glomeruli and outer-medulla of rat kidney

The isolation of rat kidney glomeruli was conducted according to the method of Fujiwara et al. (1989) with some modifications. Briefly, Sprague-Dawley rats weighing above 200 g were sacrificed and kidneys immediately removed and placed in ice-cold Dulbecco's PBS (DPBS), pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 KH_2PO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 5.6 mM glucose. The cortex was dissected and minced to paste-like consistency by a tissue chopper with 100 um interval, and then suspended in cold DPBS. The suspension was passed successively through the 200 um and 150 um sieves, then the glomeruli were collected on a 50 um sieve which was washed with DPBS. The collected glomeruli pellets were checked for purity by light microscopy. For the isolation of kidney outer medulla, kidneys were dissected longitudinally 4-5 mm thick and the middle section of the kidney was dissected free at 4 ^{0}C.

Enzyme assays

APA enzyme activity was assayed using alpha-glutamyl-2-naphthylamide (Bachem Bioscience, Philadelphia, PA) as substrate. Specific activities are expressed as U/mg protein, where 1 U equals the hydrolysis of 1 nmole substrate per hr (Wilk and Thurston, 1990). Whole kidney, isolated kidney outer medulla and kidney glomeruli were homogenized with a polytron at 4^{0}C for 20 sec. in 0.05 M Tris-HCl buffer, pH 7.5. APA activity was measured from homogenates of the above three preparations of kidney.

Immunoblots

Immunoblots with APAAb were conducted using kidney homogenates with some modifications of the method described by Harlow and Lane (1988). Briefly, kidney homogenates or purified APA and APM from rat kidney were prepared with sample buffer containing 60 mM TrisHCl, 2% SDS, 100 mM DTT and 0.01% Coommassie brilliant blue. The kidney samples were boiled for 5 min (purified APA or APM was boiled for 1 min followed by SDS PAGE) and centrifuged for 10 min. The resulting supernatants of kidney protein and the purified APA and APM samples were separated by 10% SDS PAGE (40 ug protein/kidney sample, 1 ug protein/purified APA or APM) and then transferred to the PVDF membrane (Immobilon membrane. Millipore. Bedford, MA) in the presence of a transfer buffer containing 25 mM Tris base, 192 mM Glycine and 15% methanol at 70 volts for one hr. The membrane was treated with a blocking buffer containing 5% nonfat dry milk and 0.02% Na azide with agitation at 37°C for one hr. The blocked membrane was washed with PBS 5 min × 2 and then incubated with either APAAb (1:2000 dilution) or preabsorbed serum (APAAb preabsorbed with purified APA 5 ug/ml at 4°C overnight) at 4°C overnight. The membrane was washed with PBS 5 min X 4 and then incubated with peroxidase-labeled goat secondary antibody against rabbit IgG at 37°C with agitation for 3 hrs. The membrane was washed with PBS 5 min X 4 and incubated with 10 ml 0.05M TrisHCl (pH 7.6) containing 6 mg diaminobenzdine and 10 ul 30% hydrogen peroxide for about 5 min. The membrane was then washed with PBS and dried. Immunoblots of outer medulla and glomeruli protein samples were studied by the same method.

APA anticatalytic assays

APA was purified from rat kidney in collaboration with Dr. Sherwin Wilk. APA activity closely copurified with APM, but both activities could be separated by APM affinity column (Leu-Gly-sepharose) as the last purification step. The resulting APA peak was free from traces of APM and was used for the production of anti-APA antiserum. The kidney homogenate was prepared by taking fresh kidney tissues into 0.05 M TrisHCl pH 7.5 and homogenizing with polytron homogenizer driven at 800 rpm at 4°C. The enzyme assay was conducted immediately. The specific enzyme activity of APA in rat kidney homogenate was assayed in the absence and presence of different dilutions of the anti-APA antibody (1:100, 1:300, 1:1000, 1:3000, 1:10000). The assay was repeated 5 times.

Immunocytochemistry

Male Spraque-Dawley rats were processed for immunocytochemistry according to the method described elsewhere (Healy and Wilk, 1993) with some modifications. Briefly, rats were perfused transcardially with Zamboni's fixative (2% paraformaldehyde, 15% picric acid, 85% 0.12 M phosphate buffer pH 7.4) under pentobarbital (50 mg/kg, i.p.) anesthesia. Rat tissues of pituitary, kidney, adrenal, intestine, lung and liver were removed, postfixed in the same fixative at 4°C for one week and then sequentially transferred through PBS buffer containing 12% and 16% sucrose for 2 hrs, and then 18% sucrose overnight. Cryostat sections of tissues were collected onto cold gelatin-

coated slides. Sections were preincubated with 2% normal goat serum in PBS containing 0.3% Triton X-100 30 min followed by overnight incubation with immune sera diluted (1:5000) in PBS containing 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA). Sections were rinsed with PBS and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) in a 1:222 dilution with PBS-Triton X-100 for 45 min. The sections were washed with PBS and incubated with streptavidin-horseradish peroxidase conjugate for 45 min. The peroxidase reaction was developed by treating the sections with a freshly prepared 0.05% solution of 3,3'-diaminobenzdine (Sigma chemical Co., St. Louis, MO) containing 0.003% hydrogen peroxide in a 50 mM phosphate buffer, ph 7.4, 5 to 10 min. The sections with or without cresyl violet counter staining were dehydrated in alcohol, defatted in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Sections were viewed and photographed with a Zeiss microscope.

In situ hybridization

The partial cDNA of APA from rat kidney previously published (Song et al., 1994) was subcloned into pBluescript II SK +/-. For riboprobe synthesis, sense and antisense ^{35}S-riboprobes were transcribed from the 450 bp cDNA in TA cloning vector (for antisense synthesis) and in pBluescript (for sense-riboprobe synthesis) by using ^{35}S-CTP (DuPont Wilmington DE) and commercially available reagents (New England Biolabs, Inc., Beverly, MA). For antisense riboprobe synthesis, the plasmid was linearized with EcoRI which cleaved the EcoRI site in the 5' end of the cDNA, and transcription was catalyzed by T7 polymerase. For sense riboprobe synthesis, the APA

cDNA in pBluescript was linearized with KpnI in the 3' end of the cDNA and transcription was catalyzed by T3 polymerase.

In situ hybridization was performed according to the method of Valentino et al. (1987) and Sambrook et al. (1989) with some modification. Briefly, freshly frozen rat kidney, pituitary and intestine were cryostat sectioned at 15 microns at -20°C and melted on to cold silane-coated slides. After 3% paraformaldehyde fixation, the slides were vacuum dried and stored at -20°C for future hybridization. Prewarmed tissue sections were treated sequentially in the buffer containing 1 M Tris pH 8, 0.5 M EDTA at RT for 5 min, and buffer containing 5 M NaCl, 1 M Tris at RT for 10 min. Sections were prehybridized in a prehybridization buffer containing 1.2 M NaCl, 40 mM PIPES pH 6.7, 2X Denhardt's Solu., 2 mM EDTA, 0.1% total RNA, 0.05% tRNA, 0.1% ssDNA, 0.1% NaPPi in presence of 50% formamide and 0.1 M DTT in a humidified chamber at 50°C 2 hrs. The sections were then hybridized with ^{35}S-riboprobes (1 x 10^7 cpm/ml buffer/slide) in the same prehybridization buffer using 0.01% total RNA, 0.02% ssDNA instead of 50% formamide and 10% dextran sulfate present in above prehybridization buffer at 50°C overnight. The solution containing the riboprobes was removed and sections treated sequentially with the following buffers: buffer containing 0.3 M NaCl, 10 mM Tris pH 8, 0.05% NaPPi at RT for 10 min X 2; RNase buffer containing RNase 30 ug/ml, 0.3 M NaCl, 10 mM Tris pH 8, 1 mM EDTA at RT for 30 min; buffer containing 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 0.05% NaPPi, 10 mM DTT at RT for 10 min; buffer containing 75 mM NaCl, 10 mM Tris, 1 mM EDTA, 10 mM DTT at 50°C for 30 min; and sections were rewashed in the last buffer at 50°C overnight. Sections were rinsed sequentially in 70% ethanol/0.3 M NH4Ac, 90%

ethanol/0.3 M NH4Ac, and then 100% ethanol 2 min each RT. The air dried slides with or without counterstaining by cresyl violet were dipped in liquid emulsion and exposed for a period of one month.

Histochemistry

The histochemical studies of APA activities of rat kidney were conducted by using the method of Lojda and Gossrau (1980) with some modification. Briefly, fresh frozen kidneys were sectioned at 20 um and mounted on to gelatin-coated slides. The slides were vacuum dried and fixed in chloroform : acetone (1:1) fixative for 2 min. The air dried sections were then preincubated with 0.1 M sodium phosphate buffer (PH 7.2) at 26°C for 30 min. After removal of the preincubation buffer, the sections were incubated with substrate solutions of H-Glu-4MBNA (0.4mg/10 ml 0.1 M Na phosphate buffer pH 6.8, which contained 1.5 mM $CaCl_2$, 0.003% N, N- dimethylformamide and 0.01% NaOH) at RT for 30 min. The slides were then rinsed with water briefly, air dried, and coverslipped with mineral oil. The sections were examined under light microscopy.

Immunofluorescence

Freshly frozen rat kidneys were cryostat sectioned and collected on to cold slides coated with silane. After 3% paraformaldehyde fixation for 5 min, the slides were rinsed in PBS for 2 min X 2 and passed sequentially through H_2O, 50% ETOH, 70% ETOH, 95% ETOH and 100% ETOH one min each. The vacuum dried sections were then

preincubated with 2% normal goat serum in PBS containing 0.3% Triton X-100 RT 30 min followed by incubation with APAAb (1:500 dilution) in PBS containing 0.1% Triton X-100 and 0.1% BSA at 4°C overnight. Sections were rinsed with PBS 5 min × 4 and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories)/PBS-Triton X-100 (1:222 dilution) at RT 45 min. The sections were washed with PBS 5 min × 4 and incubated with fluorescein-avidin reagent/PBS-Triton X-100 (1:100 dilution) at RT for an hr. The slides were rinsed with PBS 5 min × 4 and air dried. The immunofluorescent labeling of APA in the kidney sections of rat was examined with a fluorescent-microscope.

RESULTS

Characterization of kidney APA

Since kidney was the source of purified APA from which the APAAb was made and there are high levels of APA activity in the kidney (Lojda and Gossrau, 1980), immunoblots of kidney homogenates, purified rat kidney APA or APM were conducted with APAAb (1:2000 dilution). Kidney homogenates yielded two bands (Fig.1), a major band M_r~136 kDa, a smaller band M_r~101 kDa. Purified rat kidney APA yielded a major single band of APA M_r~126 kDa. Purified APM was also faintly labeled by APAAb as a single band of M_r~120 kDa. The immunolabeling was completely blocked by preabsorption with 5 ug/ml of purified APA. Based on the immunoblots, there are a number of possibilities regarding the multiple bands: 1. The smaller bands may be degradation products of APA. 2. There may be multiple isoforms of APA. 3. APAAb

may cross-react with other proteins in the kidney. Immunoblots in presence of a battery of protease inhibitors (Huo et al., 1991) did not alter the labeling pattern, suggesting that kidney APA was not degraded during the experimental procedure. Only one form of APA has been identified by molecular cloning (Wu et al., 1990, Wu et al., 1991, Song et al., 1994), but the existence of additional isoforms cannot be ruled out. Immunoblots with APAAb (1:1000 dilution) and freshly prepared samples of total kidney, outer medulla and glomeruli yielded three bands in each tissue with M_r~136 kDa, M_r~129 kDa and M_r~107 kDa. (n = 5, Fig.2). Immunoblots of these samples with APAAb preabsorbed with purified APM or DPPIV (1 ug/ml) demonstrated that the labeling of the M_r~129 kDa band was blocked by purified APM and DPPIV blocked the labeling of the M_r~107 kDa band. Preabsorption of APAAb with APM and DPPIV simultaneously resulted in staining of the only M_r~136 kDa band. These experiments indicate that the APAAb cross-reacts with APM and DPPIV of the kidney. Thus the major band with M_r~136 kDa represents labeling of APA, the M_r~129 kDa band is APM and the M_r~107 kDa band is DPPIV. While the smaller 126 kDa band of purified APA from rat kidney may be the truncated form of APA due to the process of autolysis, a method used in the first step of purification to solubilize APA from rat kidney membrane.

In order to further characterize the rabbit antiserum against the purified APA from rat kidney, anticatalytic assays against rat kidney homogenate were conducted with different dilutions of APAAb. The maximum anticatalytic activity of the antiserum was approximately 70% of total activity. The mean IC_{50} of APAAb dilution in 5 repeated anticatalytic assays was 1:242 (Fig.3). Suggesting that APAAb binds to APA of rat

kidney in the area of catalytic site.

Immunoreactivity with APAAb (1:5000 dilution) for the kidney demonstrated that APA was primarily located within the lumen of proximal tubules. Preabsorption of APAAb with 1 ug/ml of APM or DPPIV had no effect on the immunostaining pattern of APAAb in the kidney (Fig.4). Preabsorption of APAAb with 1 ug/ml APA completely blocked the staining. APA immunocytochemical-staining within glomeruli was less intense and more diffuse. However, previous histochemical studies suggest that glomerulus has high APA activity (Kugler, 1981., Kugler, 1982a). Likewise the labeling intensity of APA band in the immunoblots above was strongest in the kidney glomeruli, and almost the same labeling intensity was present in both kidney outer medulla and kidney homogenate. Likewise studies on APA enzyme activities of kidney homogenate, kidney outer medulla, and isolated glomeruli of kidney (Table 1, Fig.5) indicate that the highest APA activity was present in kidney glomeruli. The specific enzyme activity (SEA) of APA in the isolated glomeruli was about 4 fold higher than that in both kidney homogenate and kidney outer medulla. We therefore conducted immunofluorescent studies using sections of fresh frozen kidney that were fixed briefly on the slides. There was intense APA staining within the glomeruli in addition to the labeling in the brush border of proximal tubules (Fig.6). These results suggest that the procedure of perfusion-fixation attenuated the APA staining of the glomerulus. Close examination of the immunofluorescent staining of glomerular with APAAb indicated that the staining was concentrated primarily within mesangial cells. The stellate appearance of mesangial cell with multiple processes terminating on the outer side of glomerular capillaries was clearly visible (Fig.6. arrow). Endothelial cells lining the capillaries were

not labeled and staining of epithelial cells was not clear.

In situ hybridization studies of the kidney indicated that mRNA of APA was highest in the outer stripe of the outer medulla (Fig.7.C.), where the S3 segment of proximal tubules is located. Cortical labeling was punctuate, with the size and distribution suggestive of glomeruli. High power view of in situ hybridization indicated that the cortical labeling was primarily within glomeruli (Fig.8). The labeling within the glomeruli was concentrated in patches and absent in majority of the cells. This pattern of labeling suggests a localization to mesangial cells as was seen in immunofluorescence image of glomeruli (Detlef, 1987). No labeling was found in the proximal tubules, distal tubules or collecting ducts. Labeling was only found in the terminal portion of afferent arteriole and renal vasculatures. There was no detectable labeling in the inner stripe of outer medulla or inner medulla. Control hybridization with sense riboprobes produced only diffuse background labeling (Fig.8.G.).

APA immunostaining and APA mRNA in other tissues

APA immunostaining was located in the microvessels of posterior pituitary and the sinusoids of anterior pituitary (Fig.9.A.B.C.). There was no APA immunostaining present in the intermediate pituitary. In the lung, alveolar capillaries were labeled by APAAb, while the epithelial cells (type I cell) on the surface of the alveoli were not labeled (Fig.10. arrow). There was no APA immunostaining in the bronchioles (Fig.10. asterisk). APA was also located in the cell membranes of heptocytes and sinusoids of the liver, while the central vein of the heptocyte was not labeled (Fig.11.E.F.). APA

immunostaining was present in the sinusoids of all layers of the adrenal cortex, but APA was not found in the adrenal medulla (Fig.11.A.B.). There was intense APA labeling on the luminal side of the villi of the intestine, namely the apical surface of the simple columnar cells of the intestine (Fig.11.C.D.).

In situ hybridization studies with APA antisense ^{35}S-riboprobes demonstrated that APA mRNA was abundant in the anterior pituitary (Fig.7.A.) and in the lumenal side of the intestine (Fig.7.E. arrow). There was diffuse background labeling in the same two tissues with sense APA riboprobe (Fig.7.B.F.). Tissues with lower APA levels such as liver, adrenal and lung were not labeled under the in situ hybridization conditions used here.

DISCUSSION

Beside the well known endocrine RAS, the presence of components of the RAS in a variety of tissues such as in the brain, pituitary, heart and adrenal (Lilly et al., 1985; Campbell, 1987; Unger et al., 1988; Moffett et al., 1987) indicate the possible paracrine effects of the RAS in both central and peripheral BP regulation. In peripheral tissues, AII is the most potent angiotensin peptide (Skeggs, 1984). APA converts AII to AIII (Nagatsu et al., 1965; Abhold and Harding, 1988). APA has also been found in many tissues of rat by the method of immunocytochemistry and histochemistry (Kugler, 1981; Kugler, 1982a; Lojda and Gossrau, 1980; Li et al., 1993). APA could therefore also be considered a component of the endocrine and paracrine RAS. With the production of the experimental tools of APAAb and APA cDNA, the cellular

localization of APA in different tissues of rat was further studied.

APA activity is high in the kidney (Lojda and Gossrau, 1980). APA was purified from rat kidney and antiserum against the purified APA (APAAb) was produced in rabbits. In an effort to characterize APA from rat kidney, immunoblots of kidney homogenates, purified rat kidney APA or APM were conducted with the APAAb (1:2000 dilution). Kidney homogenate yielded two bands with APAAb labeling: a major band of M_r~136 kDa, a smaller band of M_r~101 kDa. While purified APA of rat kidney yielded a single band of M_r~126 kDa and purified APM had a faint band of M_r~120 kDa. The fact that preabsorption of purified APA with APAAb completely blocked the APA labeling and the two bands of APA in kidney homogenate suggests the possibility of cross-reactivity of APAAb with other proteins of rat kidney as mentioned above. The smaller band of purified APA from rat kidney compared to the APA band of kidney homogenate in immunoblots may be explained by the method of autolysis used in the first step of APA purification to solubilize APA from the kidney membranes (data from Dr. S. Wilk). APA is known to be an ectoenzyme with a short cytoplasmic tail and a single transmembrane spanning domain (Wu et al., 1990; Wu et al., 1991). The solubilized form of APA is presumably a truncated form of the enzyme lacking the membrane anchor, whereas the native enzyme from kidney homogenates would be a larger membrane-bound form. Further studies of immunoblots for the whole kidney, kidney outer medulla, and kidney glomeruli with preabsorption of APM and dipeptidyl peptidase IV (DPPIV), a enzyme cleaves preferentially N-terminal aminoacyl-Pro dipeptides from different polypeptides, indicate that APAAb cross-reacted with APM and DPPIV of rat kidney, where APA was the major band of M_r~136 kDa, APM was the

band of M_r~129, and DPPIV was the band of M_r~107 kDa.

The anticatalytic assay of kidney homogenate with diluted APAAb and the immunocytochemistry of kidney section with APAAb 1:5000 dilution indicate that the APAAb binds APA of rat kidney. Although there is cross-reactivity with the APM and DPPIV of the rat kidney as shown in the immunoblots above, this may be the result of either contamination during the purification procedure or the presence of identical epitopes in these three enzymes; there is 30% similarity between the APA and APM at the amino acid level (Olsen et al., 1988; Wu et al., 1990). When greater dilutions of APAAb were used for immunocytochemistry, preabsorption of APM and DPPIV had no effect on the labeling of APA band, suggesting that the APAAb was selective for the APA at high dilutions.

Immunofluorescent labeling and in situ hybridization of rat kidney indicate that the distributions of APA immunostaining and APA mRNA in the nonperfusion-fixed sections of rat kidney were present in the glomeruli and descending segment of proximal tubules. The absence of APA immunostaining in the glomeruli of the perfusion-fixed kidneys may be the result of diminished staining of vascular elements within glomeruli with perfusion-fixation. However, APA mRNA was not detected within early proximal tubules where a high levels of APA immunoreactivity was found. This difference could represent the existence of different APA isoforms. However there is no independent evidence in support of this. In addition the riboprobe used for in situ hybridization would be expected to recognize transcripts from all APA isoforms since the essential zinc-binding domain was included within the probe. The more likely

possibility is that there is simply low turnover of the APA protein within proximal tubules and thus low levels of APA mRNA.

AII stimulates the reabsorption of Na^+ in the lumen of proximal tubules (Campese and Hsueh, 1983). AII receptors are concentrated in the apical surface of the renal proximal tubules (Mujais et al., 1986). The presence of APA in the brush border of proximal tubules suggests its role of either regulating AII stimulated sodium reabsorption by affecting the $t_{1/2}$ of AII or acting as a general peptidase for the reabsorption of amino acids in the lumen of renal tubules. APA immunoreactivity was also seen within JG cells. The co-localization of AII receptor (Chansel et al., 1992) and APA in the JG cell suggests that APA may indirectly regulate the renin release by inactivating AII locally to block the negative feedback effect of AII on renin release (Garrison and Peach, 1990; Antonipillai et al., 1989).

The high levels of APA mRNA and immunoreactivity associated with kidney glomeruli are also consistent with the highest APA activities there. The APA immunofluorescence and in situ hybridization results within the glomeruli indicate that APA is localized within mesangial cells. Mesangial cells are known to contract in response to AII stimulation (Hall, 1986; Levens, 1981), which results in reduced capillary permeability following by volume retention. AII receptors are located in the mesangial cells (Chansel et al., 1992). The presence of APA in mesangial cells may be important in modulating the effects of both circulating and locally formed AII on the glomerular filtration.

The RAS plays an important role in the regulation of sodium balance (Garrison and Peach, 1990). In addition to its effects on the kidney fluid homeostasis just described above, AII and AIII stimulate zona glomerulosa of the adrenal cortex to increase the synthesis and release of aldosterone. AII also stimulates cortisol secretion from zona fasciculata cells and catecholamine release from adrenal medulla. AII receptors are present in the zona glomerulosa, zona fasciculata and cell membranes of adrenal medulla (Jimenez et al., 1991; Aguilera, 1992; McMillian et al., 1992). APA immunoreactivity was associated with sinusoids of the adrenal cortex, suggesting that APA may be involved in regulating the effects of AII on the production and secretion of adrenal steroid hormones. Although no APA was detected in the adrenal medulla, the fact that blood in the arterial branches of adrenal capsules drains into the medullary vein through the three zones of adrenal cortex and that cortisol in the adrenal blood flow influences the activity of catecholamine system in the chromaffin cells of adrenal medulla (Garrison, and Peach, 1990), suggests that APA in the adrenal cortex may regulate indirectly the synthesis and secretion of epinephrine in the adrenal medulla.

In addition to increasing the synthesis of ADH in the supraoptic nucleus of the brain, AII stimulates the release of ADH from posterior pituitary (Ganong, 1984). APA may regulate indirectly the release of ADH by metabolizing circulating or locally produced AII there. AII also stimulates the AII receptors of anterior pituitary for the release of prolactin and adrenocoticortrophic hormone (ACTH) (Healy et al., 1992; Saavedra, 1992). The sinusoids of hypophyseal portal system connect the capillaries of median eminence to the cells of anterior pituitary so that APA in the sinusoids of anterior pituitary could regulate the effects of AII on the release of anterior pituitary

hormones. Therefore, it is suggested that APA located in the anterior and posterior pituitary may regulate the endocrine and paracrine effects of AII on the synthesis and release of variety of pituitary hormones.

The lung is highly vascularized. AII has weak vasoconstrictor effects on the lung (Garrison and Peach, 1990). AII receptors are known to be associated with endothelial cells and smooth muscle cells of the blood vessels in the lung (Catt et al., 1984). ACE is located at the endothelial cells of the lung (Stalcup et al., 1982). ACE activity (mU/mg protein) of the lung is about 10 fold higher than that of other tissues including brain, kidney, and aorta (Sattar et al., 1985). The presence of APA in the capillaries of the lung may therefore protect the lung interstitium from the effect of AII by regulating the levels of AII in the lung.

In addition to being the primary organ for angiotensinogen production, the liver exhibits a number of responses to AII including stimulation of glycogenolysis and gluconeogenesis, and inhibition of fatty acid synthesis (Campanile et al., 1982). AII stimulates angiotensinogen production (Garrison and Peach, 1990). The sinusoids of the liver connect the portal vein and hepatic artery from periphery of the lobule to the central vein in the middle of the lobule. AII receptors are located in the cell membranes of liver (Jimenez et al., 1991; Cazaubon et al., 1993). The localization of APA immunoreactivity in the hepatocyte membranes indicates that APA may provide a immediate regulation of AII effects on the physiological functions of liver.

There are no reports of AII receptors in the intestine. The high levels of mRNA and enzyme protein of APA on the columnar cells of intestine suggest its active role as a general peptidase there for the absorption of amino acids.

Therefore, the broad tissue distribution of APA suggests its importance in the regulation of the endocrine and paracrine effects of AII on the physiological functions of a variety of tissues. APA may regulate the effects of AII by influencing its levels in peripheral tissues.

Fig.1. Immunoblot of kidney APA. Kidney homogenates, purified kidney aminopeptidase A (APA), and membrane aminopeptidase M(APM) were separated on a 10% SDS PAGE, transferred to a immobilon membrane and incubated with either APAAb (1:2000 dilution) or APA immune serum preabsorbed with the purified APA (5 ug/ml). The antibody-antigen complex was visualized with a goat anti-rabbit horseradish peroxidase-coupled antibody, with diaminobenzdine as substrate. Note that with APAAb, kidney homogenates yielded a major band of Mr 136 kDa, a second band of Mr 101 kDa and diffuse lower molecular weight material. Purified APA yielded a single band of Mr 126 kDa, whereas purified APM yielded a faint band of Mr 120 kDa. With APA immune serum preabsorbed with purified APA, the labeled bands of the kidney and the purified APA or APM were almost completely absent.

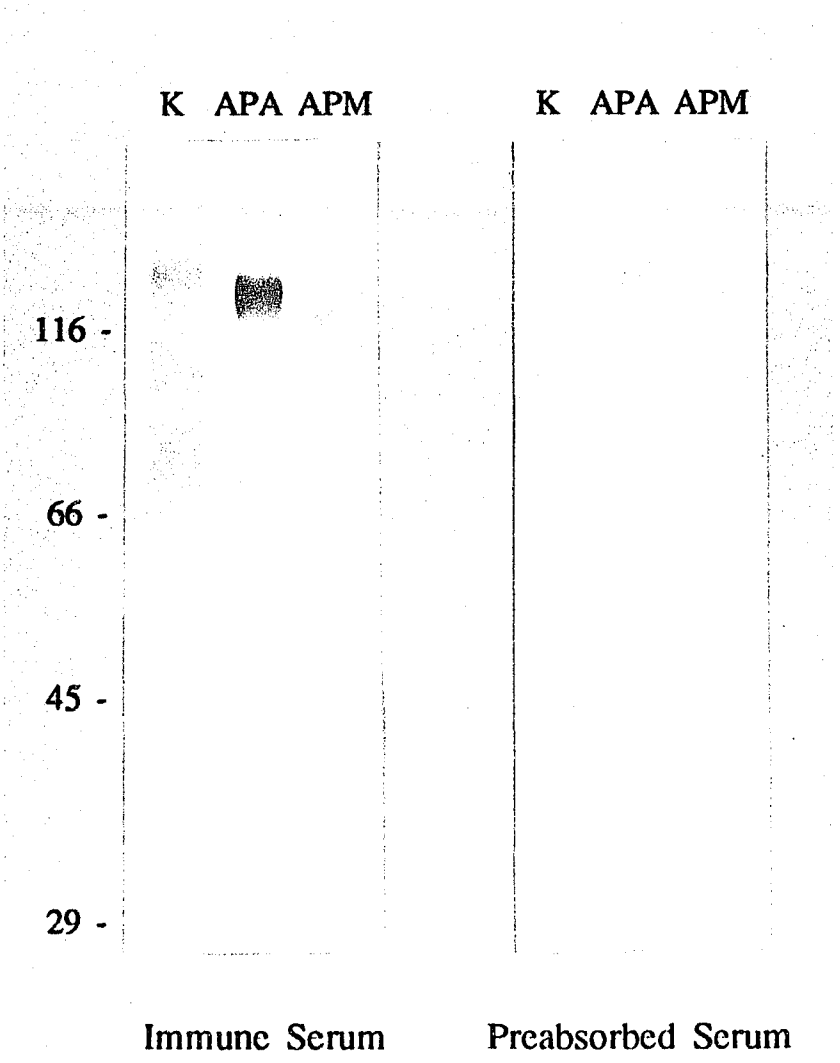

K APA APM
K APA APM
116 -
66 -
45 -
29 -
Immune Serum
Preabsorbed Serum

Figure 1

Fig.2. Immunoblots of rat kidney APA. Purified APA (A) and protein samples from homogenates of total kidney (K), outer medulla (M), and isolated glomeruli (G) were separated by SDS-PAGE under reducing conditions, transferred to immobilon membrane, and immunostained with APAAb (1:1000 dilution). Size of labeled bands was determined by comparison to molecular weight markers (29-205 kDa). The staining pattern with control immune serum was compared with that with immune serum preabsorbed with either 1 ug/ml dipeptidyl peptidase IV (+DPPIV) or with 1 ug/ml aminopeptidase M (+APM). The size of the putative APA protein here (arrow) was 136 kDa.

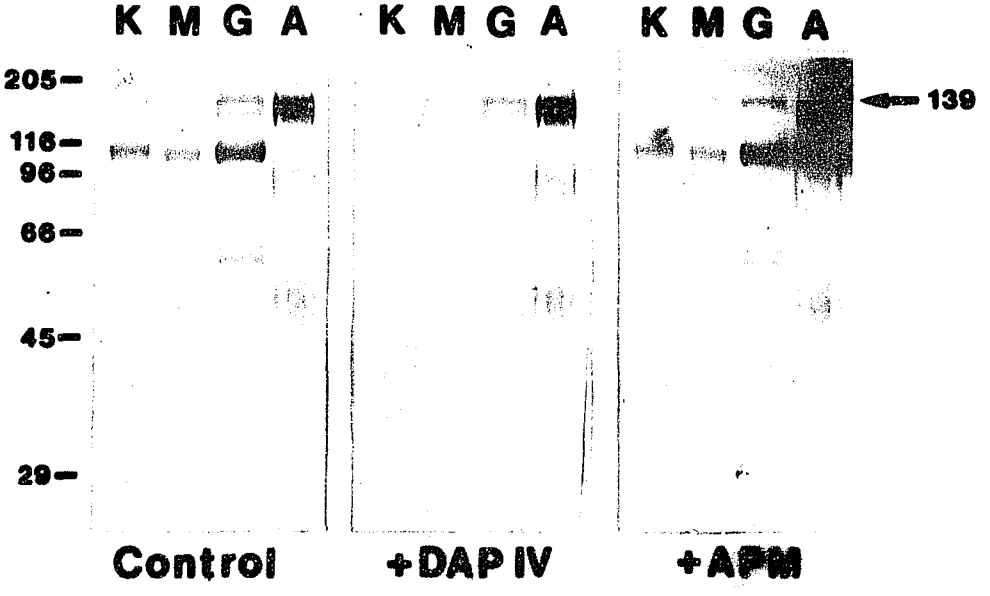


Figure 2

Fig.3. Anticatalytic immunoinhibition assay of kidney APA activity with anti-APA anti serum. Shown is a representative experiment where kidney homogenate were incubated with increasing dilutions of APAAb and APA activity (nmol/mg/h) measured using α-glutamyl-2-naphthylamide as substrate. The dilution of APAAb that resulted in 50% inhibition of APA activity was termed the IC_{50}. In this experiment the IC_{50} was a 1:300 dilution of APAAb.

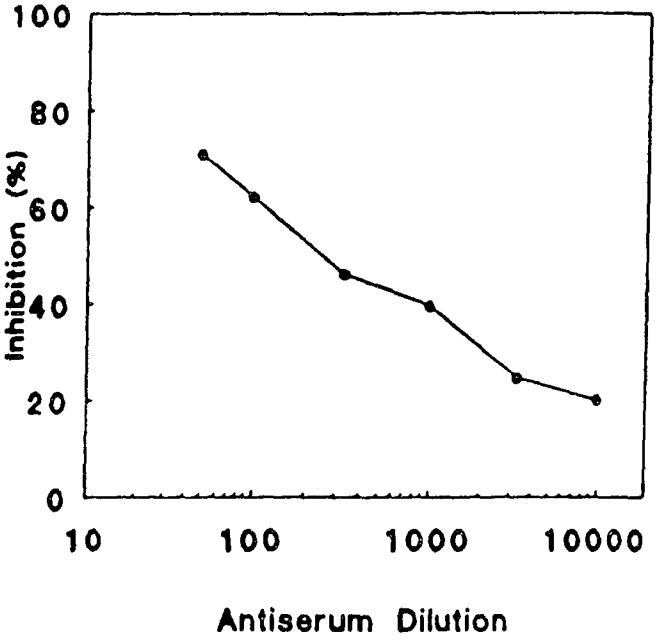

100
80
60
40
20
0
Inhibition (%)
10
100
1000
10000
Antiserum Dilution

Figure 3

Fig.4. Effects of preabsorption of the APA antiserum (1:5000 dilution) with purified APM and DPPIV on the immunocytochemical staining pattern in rat kidney. A: immunostaining with control antiserum. Note the typical staining pattern in the outer cortex, with heavily labeled proximal convoluted tubules and lighter stained glomeruli. B: immunostaining with antiserum preabsorbed with APM (1 μg/ml). Note there is no reduction in staining. C: immunostaining with antiserum preabsorbed with DPPIV (1 μg/ml). Note there is no reduction in staining. D: immunostaining with antiserum preabsorbed with APA (1 μg/ml). Note the almost complete absence of immunostaining. Bar = 100 μm.

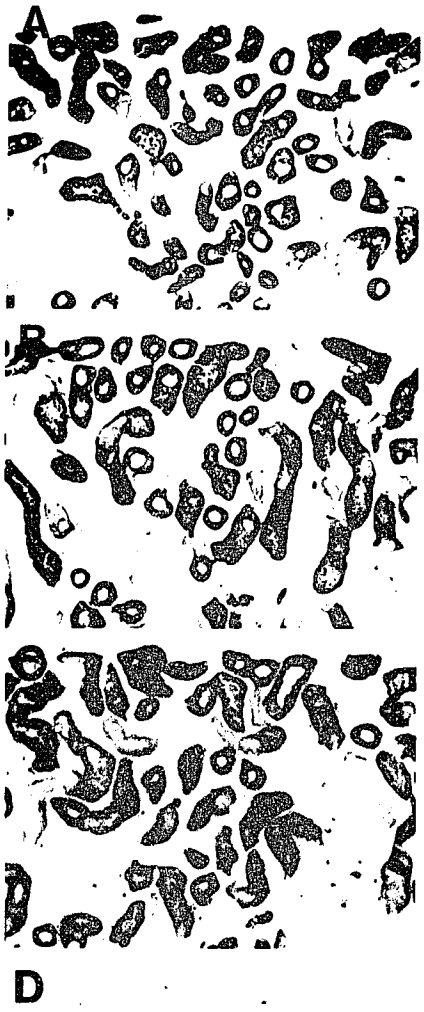


Figure 4

Fig.5. Phase-contrast photomicrograph of isolated glomeruli following sieving procedures. Shown in a cluster of glomeruli from the final 50 um nylon sieve collection.

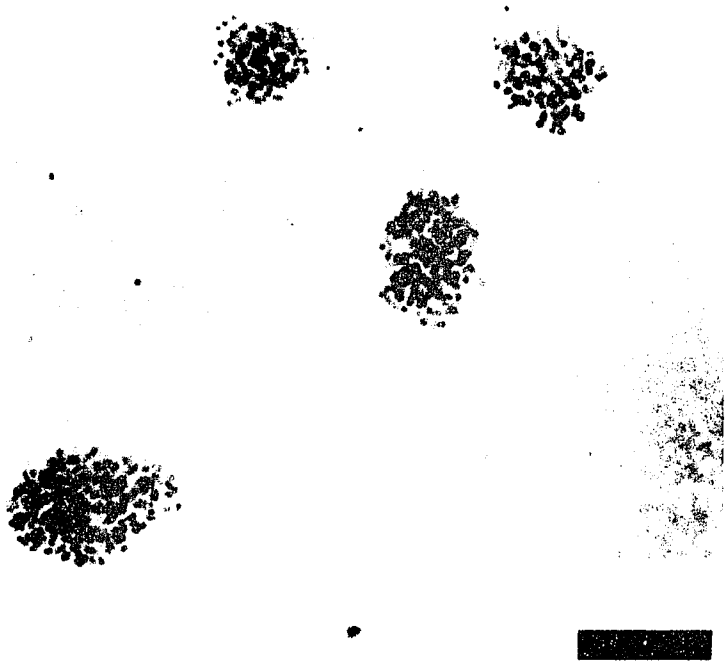


Figure 5

Fig.6. APA immunofluorescent staining within the kidney. A nonperfused kidney was cryostat sectioned, fixed on the slide, and immunostained with APAAb using an immunofluorescent method. A: Photomicrograph of APA immunoreactivity within a glomerulus. Note that APA immunofluorescence was concentrated within the mesangium, specifically within mesangial cells (arrow) and mesangial cell processes. The endothelial cell linings of capillaries were not stained (*). Bar = 25 um. B: Higher power photomicrograph of the labeled mesangial cell in A, shown by arrow. Note the concentration of immunofluorescence within the mesangial cell cytoplasm and the processes (small arrow heads) that terminate on the capillary walls. Bar = 10 um.

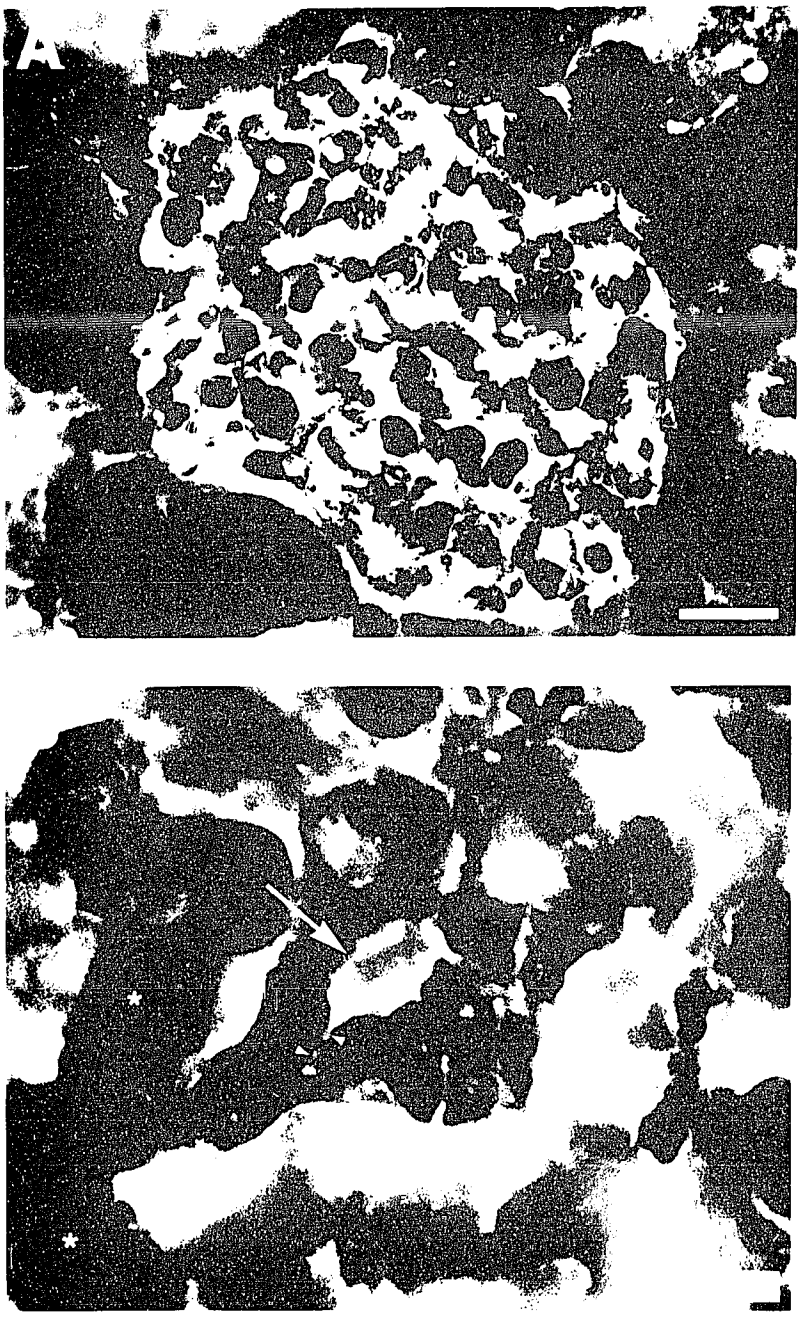


Figure 6

Fig.7. In situ hybridization of rat pituitary, kidney and intestine. The sections of the three rat tissues were incubated with either antisense (A.C.E.) or sense (B.D.F.) APA ^{35}S-riboprobe and exposed to Kodak XAR film for three days. The autoradiogram of the three tissue sections are shown below:

A. A transverse section of pituitary labeled with antisense APA ^{35}S-riboprobe. Note that the strong labeling of APA mRNA was present in the anterior pituitary (a), while the posterior pituitary (p) and the intermediate pituitary (i) were not labeled. Bar = 1 mm.

B. In situ hybridization with sense APA ^{35}S-riboprobe for the transverse pituitary section indicate no APA mRNA labeling there.

C. A transverse section of rat kidney labeled with antisense APA ^{35}S-riboprobe. The heaviest labeling was found in the outer stripe (os) of the outer medulla, extending into the cortex as the medullary rays. Cortical (c) labeling was punctuate, with the size and distribution suggestive of glomeruli. The inner stripe of the outer medulla and the inner medulla were not labeled. Bar = 2 mm.

D. The same kidney section incubated with sense APA ^{35}S-riboprobe. Note that there is no visible APA mRNA labeling in any areas of the kidney section with only a diffuse background labeling.

E. There was intense labeling of APA mRNA by antisense APA ^{35}S-riboprobe in the luminal side of intestine (arrow). Bar = 1 mm.

F. The intestine section incubated with sense APA ^{35}S-riboprobe produced a diffuse background labeling only.

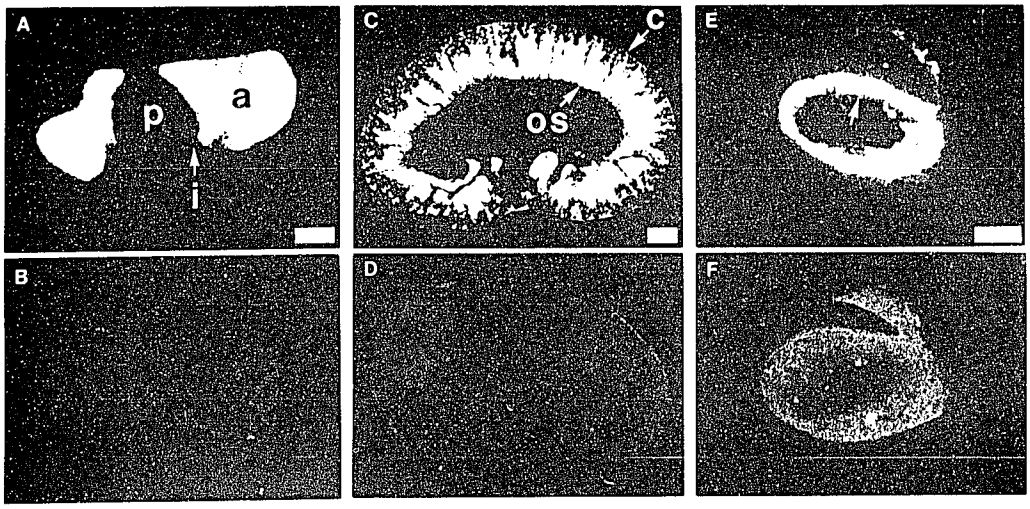


Figure 7

Fig.8. In situ hybridization of APA mRNA within glomeruli. Sections of rat kidney similar to that shown in Fig.7 were dipped in liquid emulsion and exposed for a period of 1 mo. Sections were counterstained lightly with cresyl violet. A: intermediate magnification of a labeled glomerulus surrounded by unlabeled tubules. Bar = 40 μm. B: higher power magnification of the glomerulus in A. Note that the pattern of labeling within the glomerulus is seen as clusters of silver grains and that not all cells are labeled. C-E: additional representative glomeruli showing typical labeling within glomeruli, including patches (C), cord-like patches (D), and clusters of grains situated on the extreme edge of the glomerulus, generally near the vascular pole (E). F: labeling of a glomerulus from a section incubated with an APA ^{35}S-labeled sense riboprobe. Bar = 25 μm.

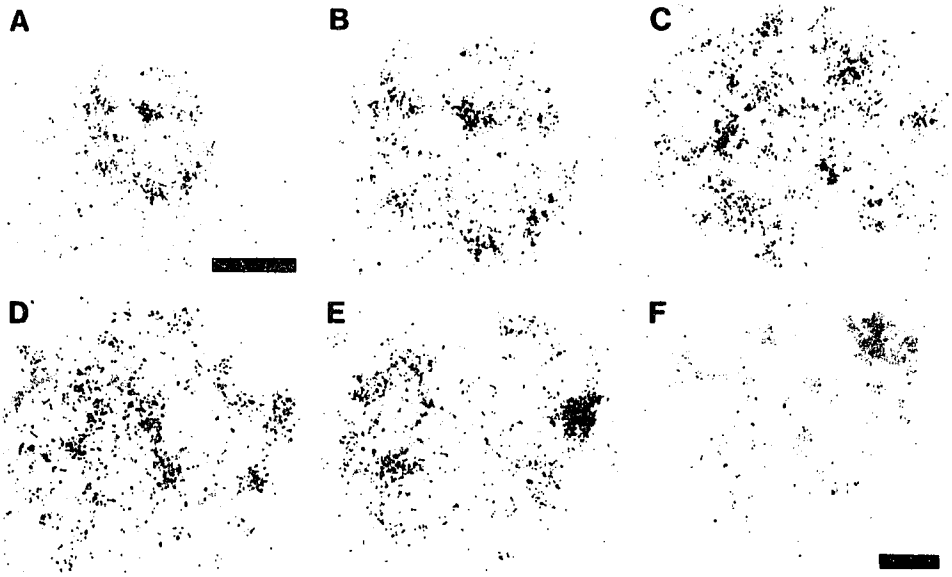


Figure 8

Fig.9. Immunoperoxidase labeling with APAAb (1:5000 dilution) plus cresyl violet counterstaining in rat pituitary. Panel A. Low power view of immunostaining in the anterior (a) and posterior (p) lobes of the pituitary. There was no APA immunostaining in the intermediate pituitary (i). Bar = 50 μm. Panel B. and C. Higher power view of the pituitary. Note APA immunostaining was present in the sinusoids of the anterior pituitary (B.). Bar = 20 μm. While in the posterior pituitary APA labeling was found in the capillaries (C.). Bar = 20 μm.

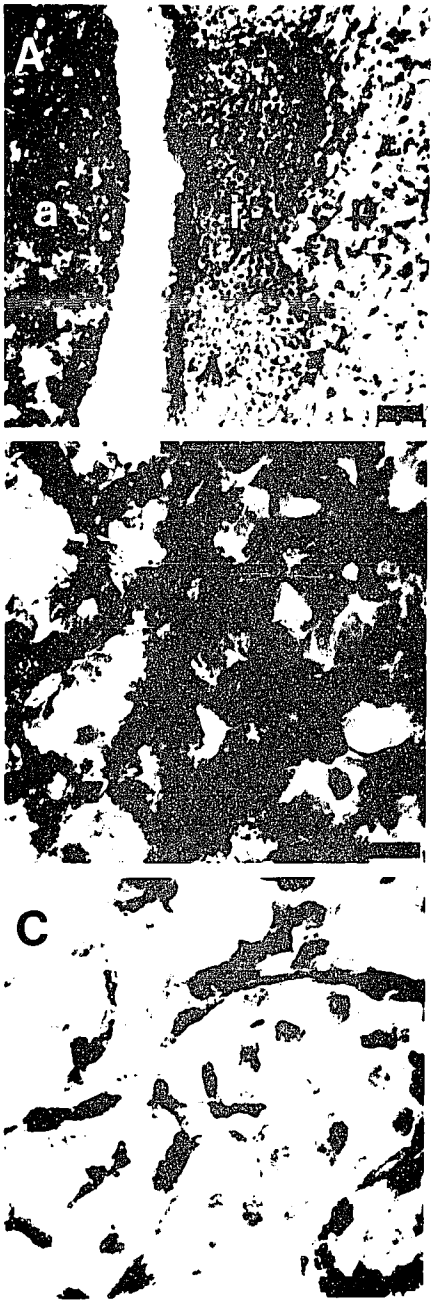


Figure 9

Fig.10. Immunoperoxidase staining of APA in the lung with APAAb (1:5000 dilution). The APA labeling in the lung was located in the capillaries of the alveolar septum. Epithelial cells (type I cell) on the surface of the alveoli were not labeled by APAAb (arrow). Note there was no clear labeling of APA in the layers of bronchus (asterisk). Bar = 50 μm.

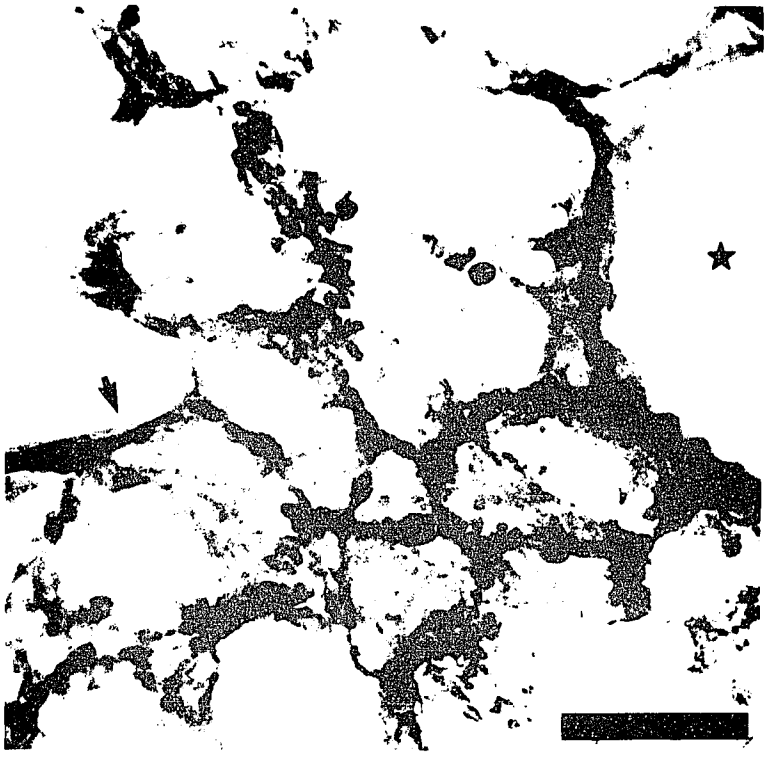


Figure 10

Fig.11. Immunoperoxidase staining of APA in rat tissues of adrenal cortex, intestine and liver with APAAb (1:5000 dilution). A. Adrenal APA immunostaining was located in the sinusoids of three zones of adrenal cortex. Bar = 50 μm. B. A high power view of APA immunostaining, together with counterstaining of the cellular nuclei, in the sinusoids of adrenal cortex. Note that the capsule of the adrenal cortex is also labeled by APAAb. Bar = 20 μm. C. Intestine. APA immunostaining is present in the apical surface of the villi. Note that the cells within the villi of the intestine also have the immunoperoxidase staining because of the endogenous peroxidase activity present within the cells of phagocytes. Bar = 50 μm. D. A high power view of APA immunostaining, in presence of counterstaining of the cell nuclei, in the apical surface of the intestine villi. Bar = 50 μm. E. Liver. APA immunolabeling, in presence of counterstaining of the cell nuclei, is found in the cell membranes of the heptocytes. The central vein of the heptocyte is not labeled by APAAb. Bar = 50 μm. F. APA immunostaining of the liver. The cell membranes of heptocytes were labeled by APAAb.

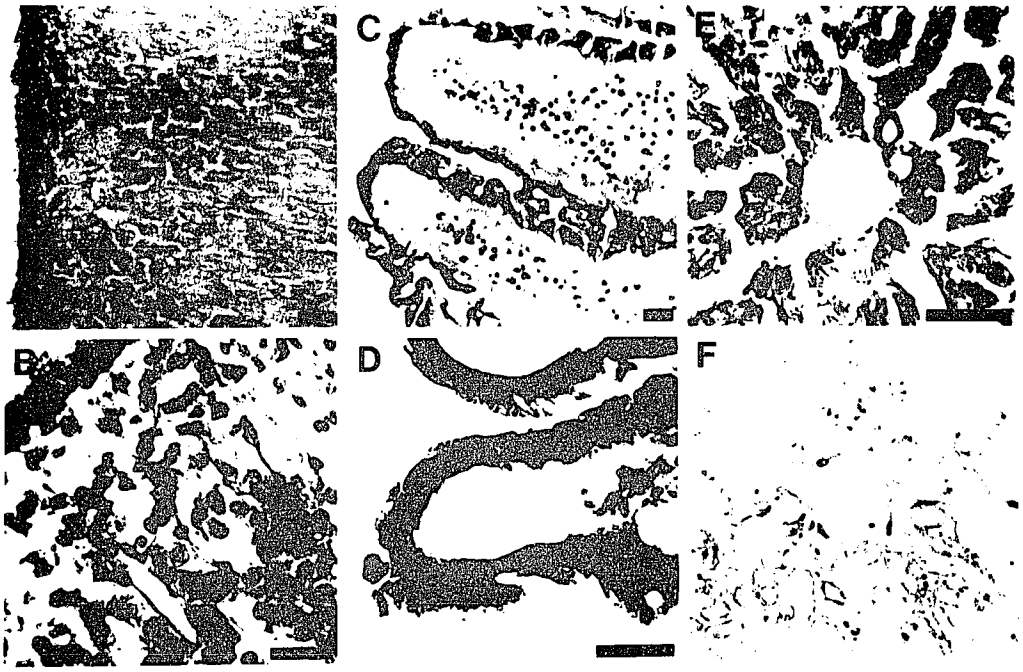


Figure 11

APA Activity in Rat Kidney

	µmole/mg protein/h	Enrichment	n
Homogenate	13.2 ± 5.2	1	5
Outer medulla	13.5 ± 2.4	1	5
Glomeruli	45.2 ± 10.6	3.4	5

Table 1. APA activity in kidney homogenate, kidney outer medulla and isolated kidney glomeruli. Specific activities are expressed as µmole of substrate cleaved per mg protein per h. Enrichment refers to the amount of APA activity in the isolated glomeruli from rat kidney divided by the APA activity in the total kidney homogenate. Values are expressed as mean ± S.E.M. Numbers under the n represent the number of independent times the experiment was prepared.

Chapter 3

CHARACTERIZATION OF AMINOPEPTIDASE A IN CEREBRAL MICROVESSELS

INTRODUCTION

It has been suggested that the renin-angiotensin system (RAS) exerts its endocrine and paracrine effects on BP and body fluid volume homeostasis in many tissues (Gross, 1968; Skeggs, 1984; Campese and Hsueh, 1983; Ben-Ari and Garrison, 1988; Skeggs, 1984; Ryan, 1982; Mendelsohn, 1985). All the components of RAS are present in the brain (Campbell, 1987). Angiotensins are thought to be neurotransmitters or neuromodulators in the brain (Moffett et al., 1987; Saavedra, 1992). The function of the brain RAS has been shown to be associated with the central regulation of blood pressure (Phillips, 1987). Localization studies indicate that both AII and AII receptors are present in many areas of brain, especially in the circumventricular organs (CVO), nucleus tractus solitarius (NTS), periventricular nuclei (PVN), and median eminence (ME) (Mendelsohn, 1984; Healy et al., 1986; Wright and Harding, 1992).

The identity of the active angiotensin peptide in the brain is still controversial. Wright et al. (1985) suggest that intracerebroventricular (icv) injected AII and AIII are equipotent in stimulating drinking and pressor reaction. It is known that both AII and AIII bind to the same receptor in the brain (Wright et al., 1990). The $t_{1/2}$ of icv injected AII is three times longer than that of AIII (Harding et al., 1986), thus the rapid degradation of

AIII in the cerebrospinal fluid (CSF) suggests that AIII may be more potent than previously considered (Wright et al., 1985; Wright and Harding, 1992). Indeed, electrophysiological studies related to water drinking and BP in the brain indicate that AIII is more potent than AII (Felix and Schlegel, 1978; Harding and Felix, 1987). AII is converted to AIII by aminopeptidase A (APA), a membrane-bound ectoenzyme that hydrolyzes the N-terminal Asp residues from AII. Amastatin, an inhibitor of APA, significantly reduced the magnitude and duration of the central pressor effects of icv injected AII, and bestatin, an inhibitor of APM, elevated both the magnitude and duration of the central pressor effects of icv injected AIII (Wright et al., 1990). Therefore, APA may be important in the central regulation of BP by converting AII to the active peptide AIII in the brain.

Previous studies in the brain have shown that APA is associated with cerebral microvessels (Bausback et al., 1988; Solhonne et al., 1987; Healy and Wilk, 1993). A number of other peptidases including aminopeptidase M (APM) are known to be associated with cerebral microvessels (Solhonne et al., 1987; Hartel et al, 1988). APA cleaves specifically N-terminal Glu or Asp residue from peptides including AI or AII and cholecystokinin-8 (CCK-8). APM cleaves all N-terminal L-amino acids residues from peptides with a preference for L-Ala. APA and APM activities are enriched in cerebral microvessels (Bausback et al., 1988; Solhonne et al., 1987), APM has also been characterized in vascular smooth muscle cells in culture (Palmieri et al., 1985; Palmieri et al., 1989). Although many peptides are known to be associated with the cerebrovasculature (Uddman and Edvinsson, 1989), the only known bioactive peptide with an acidic residue at the amino-terminus is AII. Thus APA may be a highly selective peptidase for AII in cerebral microvessels. However, APA would also be expected to degrade peptides

containing N-terminal acidic residues that were generated by other peptidases in the cerebrovasculature.

APA like immunoreactivity was reported to be associated with the adventitium of microvessels, suggesting a possible localization to pericytes (Healy and Wilk, 1993). The pericyte is an adventitial cell that is localized outside the capillaries and small arteries in the brain, and is in close contact with the basal lamina of vascular endothelial cells (Sims, 1986). The blood brain barrier (BBB) limits the accessibility of circulatory angiotensins to the central nervous system (CNS), with the exception of the circumventricular organs, areas where the blood vessels are semipermeable. The BBB is structurally composed of endothelial cells, smooth muscle cells, and pericytes (Risau et al., 1990). APA associated with cerebral microvessels may play an important role in the function of the BBB.

Therefore, since the brain RAS is related to the central regulation of blood pressure, APA may regulate the hypertensive effect of brain angiotensin peptides by metabolizing AII locally within the cerebral vasculature as well as affecting the AII levels within brain interstitium. It is important therefore to localize APA in the rat brain interstitium and the cerebral microvessels.

MATERIALS/METHODS

Experimental animals

Male Sprague-Dawley rats (175-225 g, Charles River Breeding Laboratories, Wilmington, MA) were housed on a 12 h light/12 h dark schedule and allowed free access to food and water.

Cerebral microvessel isolation

The isolation of rat cerebral microvessels was conducted using the methods of Orlowski et al. (1978) and Digilio et al. (1986) with some modification. Briefly, 10 fresh rat brains without cerebellum were homogenized in cold Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. The homogenate was centrifuged at 1000 g for 15 min. The pellets were resuspended in HBSS containing 15% dextran (M.W. 74,200), and recentrifuged at 4000 g for 20 min. The microvessel pellets were resuspended in HBSS and filtered through a 500-μm nylon mesh sieve. The filtrate containing microvessels was collected onto a 50-μm nylon mesh sieve, washed off and centrifuged at 1000 g for 15 min. The microvessel pellets in 0.32 M sucrose were centrifuged in a sucrose density gradient (1.7 M, 1.5 M, 1.2 M) at 20,000 rpm for 2 hrs. The microvessel fragments were collected from the 1.7 M fraction. The purity of the microvessel pellets was assessed by phase-contrast microscopy.

Enzyme assays

Rat brain, kidney and cerebral microvessels were homogenized at 4°C in 0.05 M Tris-HCl buffer, pH 7.5 with a teflon pestle driven at 800 rpm. APA enzyme activities were assayed according to the method described previously (chapter 2).

Immunoblots

Immunoblots of cerebral microvessels, kidney, and brain homogenates were conducted similar to the method described previously (chapter 2) with some modifications. Briefly, brain and kidney membranes were obtained by centrifugation of homogenates at 40,000 g for 20 min 2 times. The isolation of cerebral microvessels was conducted as described above. The resulting pellets were resuspended in the 0.05 M TrisHCl buffer, pH 7.5 for the preparation of protein samples as described in chapter 2. The protein samples from kidney, brain membrane and brain microvessels were separated by 10% SDS PAGE and then transferred to an immobilon membrane (PVDF). Marker protein was stained with 0.1% Coomassie brilliant blue in 10% methanol, 2% acedic acid, destained with a buffer containing 45% methanol and 7% acedic acid, washed with PBS and air dried. The membrane loaded with sample proteins was blocked by a PBS buffer containing 5% nonfat dry milk (NFDM) and 0.02% Na azide. The blocked membrane was washed with PBS and then incubated with either APA immune serum or pre-immune serum (1:1000 dilution) at 4°C overnight. The membrane was washed with PBS then incubated with peroxidase labeled goat secondary antibody (1:200 dilution). The membrane was washed with PBS and incubated with 10 ml 0.05M TrisHCl (pH 7.6) containing 6 mg diaminobenzdine and 10

ul 30% hydrogen peroxide and then washed and dried.

Immunocytochemistry

The immunocytochemistry of rat brain with APAAb (1:5000) was conducted the same way as that described in chapter 2.

In situ hybridization

The studies of in situ hybridization for APA mRNA of rat brain with APA ^{35}S-riboprobe were conducted by the same method described in chapter 2.

RESULTS

Isolation of cerebral microvessels

Previous immunocytochemical studies indicated that APA-like immunoreactivity was primarily associated with rat brain microvessels (Healy and Wilk, 1993). In order to confirm that the staining of rat brain microvessels was APA, cerebral microvessels were isolated from rat brain by using the method of Orlowski et al. (1978) with some modification. The procedure consisted of two parts: 1. passage of brain homogenate through 500 μm nylon mesh sieve and collecting on 50 μm nylon, and 2. sucrose density centrifugation. The 1.7 M fraction contained the highest concentrations of microvessels was assessed by phase-contrast microscopy (Fig.1).

APA enzyme activities in kidney, brain, and brain microvessels

APA specific enzyme activity was assayed in crude homogenates of kidney, brain and brain microvessels. APA specific enzyme activity was highest in the kidney (Table 1). The specific enzyme activity of APA in the brain microvessels in the 1.7 M sucrose fraction was 23 fold enriched over that of crude homogenate of the brain.

Immunoblots of kidney and brain tissues

Since rat kidney contains the highest levels of APA activity and was the source of purified APA for generation of the APAAb, immunoblots of kidney homogenates were compared to blots of brain membrane, brain microvessels and the purified APA from rat kidney (Fig.2). Immunoblots for each tissue with APAAb (1:2000 dilution) typically resulted in labeling of two bands: 136 kDa and 101 kDa (n = 5). A single band of 132 kDa was obtained with purified APA, consistent with it being a truncated form of APA due to the APA purification process from rat kidney as described in chapter 2. These results suggested that APA from the brain and cerebral microvessels were antigenically similar to APA of the kidney. Further studies of immunoblots with freshly prepared proteins of kidney homogenate, kidney glomeruli, and kidney outer medulla yielded three bands: 136 kDa, 129 kDa, and 107 kDa in the presence of APAAb (1:1000 dilution) as mentioned previously (chapter 2). Preabsorption with APM and DPPIV (1 ug/ml) blocked the staining of the two smaller bands, suggesting that APAAb cross-reacted with APM and DPPIV of rat kidney. The labeling intensity on a per mg protein basis was strongest in the following order: kidney homogenate, brain microvessels, and brain membrane. This rank order was

consistent with the specific activities of APA in all these tissue preparations (Table 1).

Immunocytochemistry

Immunocytochemical staining with APAAb for sections of rat brain fixed with 5% paraformaldehyde solution indicated that APA-like immunoreactivity was primarily associated with cerebral microvessels, especially associated with microvessel adventitium. To further determine the localization of APA within cerebral microvessels, an alternative fixation protocol was tested. Zamboni's fixative was used for perfusion and postfixation. In the brain, APA staining was found in the microvessel-associated pericytes. In cross-sections, pericytes could be seen with their nuclei darkly stained by cresyl-violet and their cytoplasmic processes surrounding the capillary wall labeled by APA immunostaining (Fig.3). The pericyte cell with a round nucleus bulging outside of the microvessel (Fig.3. arrow) could be distinguished from the endothelial cell with an elongated nucleus located along the lumenal side of the microvessels (Fig.3. arrow head). APA immunostaining was also located within the pia mater of the brain meninges and cells of the choroid plexus in the cerebral ventricles (data not shown here). APA immunostaining is also present in the subfornical organ (SFO), paraventricular nucleus (PVN) and median eminence (ME) (Healy and Wilk, 1993).

In situ hybridization

Studies of rat brain APA mRNA indicated that APA mRNA was located in the SFO, choroid plexus (Fig.4. A.), and in the PVN and ME (Fig.4. C.). The diffuse

background hybridization was consistent with microvessel localization but this could not be confirmed by higher resolution techniques (data not shown). Control studies with sense APA ^{35}S-riboprobe showed no labeling (Fig.4. B. D.). No labeling of neurons or glial cells was detected.

DISCUSSION

The brain contains all the components of RAS (Campbell, 1987). Angiotensin peptides are known to be involved in the central regulation of blood pressure (Phillips, 1987). APA, a specific enzyme for converting AII to AIII (Abhold and Harding, 1988), has been reported to be associated with cerebral microvessels (Bausback et al., 1988). The Km value of APA for AII is 35 uM in the cerebral microvessels compared to a Km of 130 μm in kidney (Bausback et al. 1988; Kugler, 1982). Here we confirmed that APA enzymatic activity was enriched in rat cerebral microvessels. The similar pattern of bands from immunoblots of kidney, brain, and cerebral microvessels suggests that APA is antigenically similar in these tissues. Therefore, APA in rat brain microvessels could also cleave the N-terminal aspartyl or glutamyl residues of oligopeptides including AI or AII (Kugler, 1982). AII is the only peptide containing an aspartyl residue at its N-terminus among all known bioactive peptides associated with cerebral microvessels (Uddman and Edvinsson, 1989). Thus APA may be a highly selective peptidase for AII in the cerebral microvessels. Given its substrate specificity, APA would also be expected to participate in the degradation of other biologically active peptides that were first hydrolyzed by other peptidases in the cerebrovasculature, including APM which cleaves all N-terminal L-amino acids with a preference for L-Ala; and DPPIV which cleaves preferentially N-terminal aminoacyl-Pro

dipeptides from different polypeptides (Kugler, 1982; Hartel et al., 1988).

AII is a potent vasoconstrictor and trophic factor in the vasculature (Manabe et al., 1989; Bell et al., 1989; Geisterfer et al., 1988). ACE inhibitors retard the development of hypertrophy in cerebral microvessels to a similar degree as do as the antihypertensive agent hydralazine (Hajdu et al., 1991). Studies by Stier (1993; 1989) suggested that AII stimulated the cerebrovascular lesion of stroke-prone spontaneously hypertensive rats (SHRSP), and both ACE inhibitor and AII receptor antagonist prevented the development of the cerebrovascular lesions in SHRSP. The cerebral microvessels contain AII receptors, renin and ACE (Brecher et al., 1983; Kowaloff et al., 1980; Speth and Harik, 1985). There is increasing evidence that AII is synthesized locally within blood vessels (Brecher et al., 1983; Higashimori et al., 1991; Mizuno et al., 1991). Therefore, AII, either circulating or locally formed, may be important in the development of cerebrovascular hypertrophy and lesions in hypertension.

Immunocytochemical localization of APA in cerebral microvessels was restricted to perivascular pericyte cells. It is known that within capillaries, pericytes constitute a part of the blood brain barrier (Risau et al., 1990). Pericytes are known to produce many vasoactive autoregulating agonists such as prostaglandins (Shepro and Morel, 1993). Therefore, the presence of APA in cerebral microvessel pericytes could be important in regulating the effects of angiotensins within the brain. APA associated with pericyte cells could act as a part of BBB by inactivating circulating or locally produced oligopeptides including bioactive peptide AII, limiting the entrance of bioactive peptides into the brain interstitial space, and preventing the vasoconstrictor effects of AII in the cerebral

vasculature. Interestingly, both pericytes and mesangial cells of kidney glomeruli (chapter 2) are perivascular adventitial cells with multiple processes surrounding microvessels. Both cell types are thought to be derived from the same progenitor cells (Sims, 1986). Mesangial cells are well known target cells for AII and contain APA (Song et al., 1994; chapter 2). It is not known whether pericyte cells contain angiotensin receptors.

The importance of APA in the central regulation of BP is suggested by the following factors: 1. The PVN may act as an integration center in the central regulation of BP (Wright and Harding, 1992), processing the incoming information of CVO (Fitzsimons, 1980), sending efferents to the posterior pituitary (PP) (Ganong, 1984) and to the nucleus tractus solitarius (NTS) (Rettig et al., 1986; Brattstrom, 1992), the baroreceptor reflex center (Reid, 1992). 2. It is suggested that the central elevation of BP stimulated by angiotensin peptides depends on both vasopressin release via the CVO-PVN-PP pathway and catecholamine outflow via the CVO-PVN-NTS pathway (Wright and Harding, 1992). 3. AII and AII receptor are found in the CVO, PVN, ME, and NTS (Healy et al., 1986; Healy and Wilk, 1993; Mendelsohn, 1985). 4. There is controversy as to the active angiotensin peptide in the brain. AIII is equieffective or more potent compared to AII for the central stimulation of water drinking and pressor action (Felix and Schlegel, 1978; Wright et al., 1985; Harding et al., 1986; Harding and Felix, 1987; Wright et al., 1990). 5. APA immunostaining is present in the rat brain SFO, choroid plexus, PVN, and ME (Healy and Wilk, 1993). APA is especially associated with pericytes of rat cerebral microvessels. Likewise APA mRNA is located at the SFO, choroid plexus, PVN, and ME of rat brain. Therefore, it is possible that brain APA may play an important role in central regulation of BP along with the two angiotensinergic pathways. Whereas APA may regulate

the levels of AII in the brain interstitium by acting as a part of BBB in the cerebral microvessel-pericyte, and APA may also regulate the AII levels in the CSF by inactivating or activating AII in the semipermeable blood vessels of SFO and choroid plexus of cerebral ventricles.

Therefore, in the central regulation of blood pressure, APA of the brain RAS is associated with the two central angiotensinergic pathways: AII stimulated ADH release via CVO-PVN-PP pathway, and AII stimulated sympathoexcitation via CVO-PVN-NTS pathway. APA in the pericyte of cerebral microvessels may act as a part of the BBB by activating or inactivating circulatory and locally produced AII to regulate the hypertensive effect of angiotensin peptides either in the brain interstitium or in the CSF along the two pathways.

Fig.1. Phase contrast photomicrograph of isolated microvessels following the sieving procedure and sucrose density centrifugation. Shown are microvessels from the 1.7 M sucrose fraction. Magnification. Bar = 150 μm.

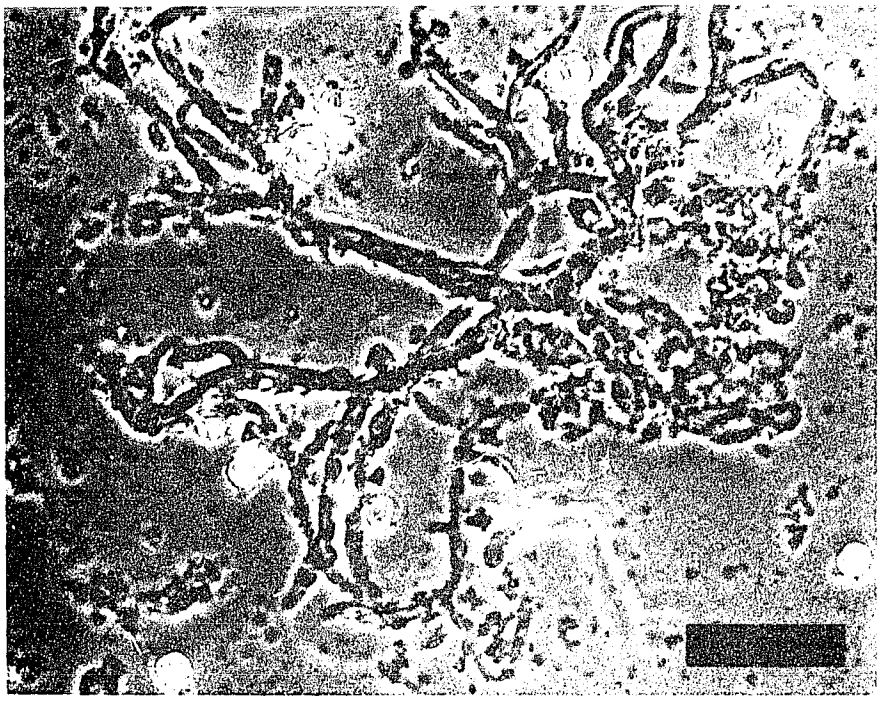


Figure 1

Fig.2. Immunoblot of purified APA and cerebral microvessels (MV), brain (B), and kidney (K) homogenates with APAAb (1:1000 dilution) or pre-immune serum (1:1000 dilution). Note that the cerebral microvessel and kidney preparations had similar patterns of labeling, with two diffuse bands, whereas no labeling was detected in the brain homogenate. The labeled bands were not seen with the pre-immune serum. Numbers (X 10^{-3}) refer to the molecular weight standards.

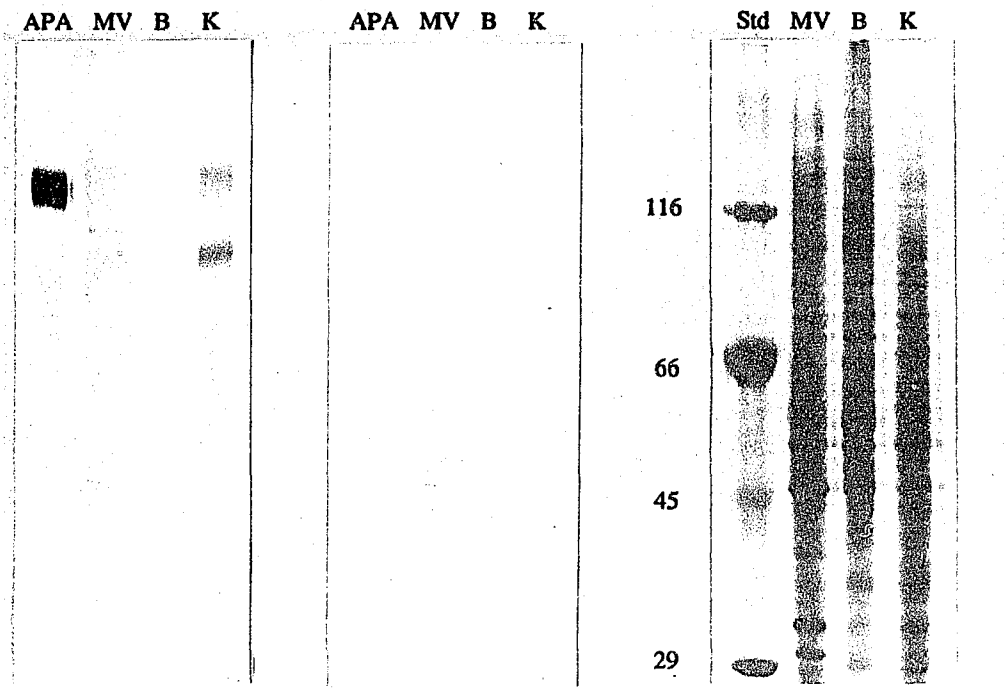

APA MV B K
APA MV B K
Std MV B K
116
66
45
29
Immune Serum
Pre-immune Serum
Coomassie blue-stained protein

Figure 2

Fig.3. Immunoperoxidase staining of cerebral microvessels with APAAb (1:5000 dilution). A. and B. APA immunostaining was located within pericytes of cerebral microvessels. Note the staining of the pericyte (arrows) bulging outside of the microvessel lumen with the cytoplasmic processes surrounding the vessel wall. The endothelial cells (arrow heads) of the microvessels with the nuclei stained by cresyl violet were located within the lumen of blood vessels and were not labeled by APAAb. Bar = 20 μm. C. and D. High power views of brain microvessel pericytes. APA immunostaining was present in the pericytes of the cerebral microvessels, where the pericytes are bulging out side of the lumen of blood vessels (C. arrow) and the nuclei of endothelial cell counterstained by cresyl violet is located within the lumen of blood vessels (D. arrow head). Bar = 10 μm.

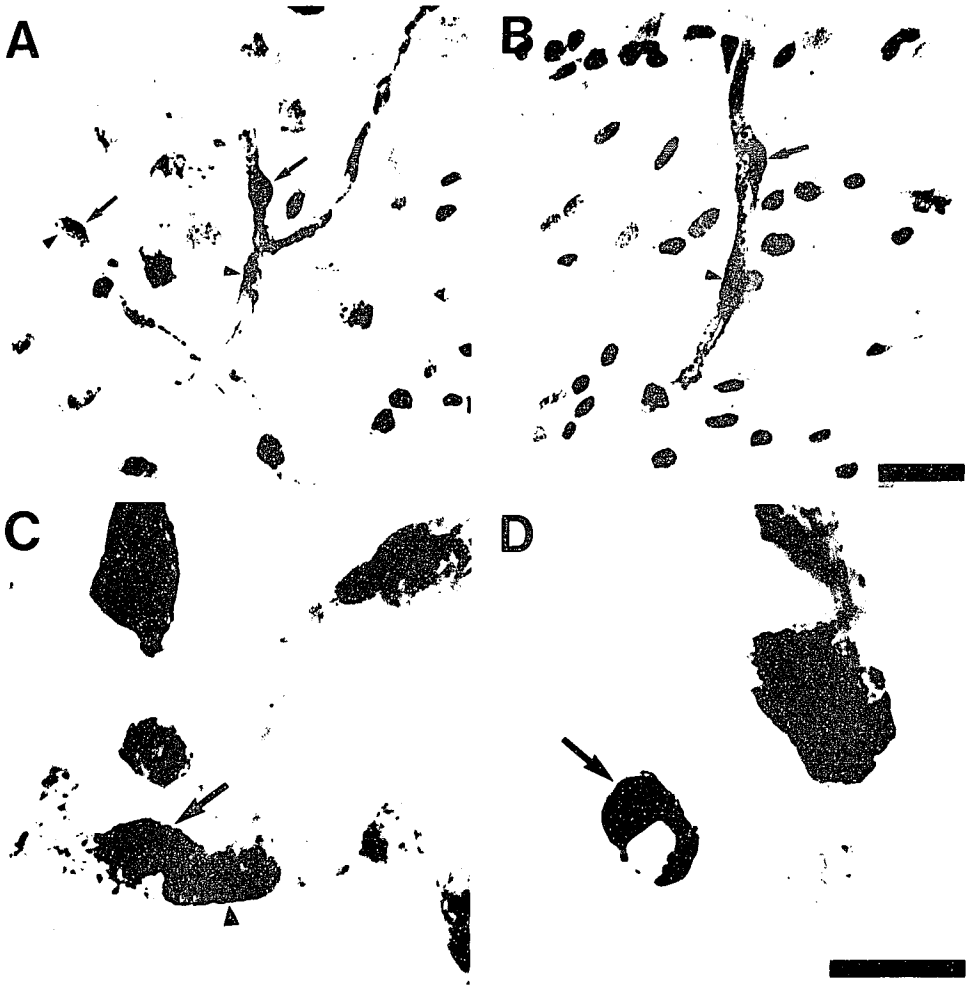


Figure 3

Fig.4. In situ hybridization of rat brain with antisense or sense APA ^{35}S-riboprobes. A. APA mRNA was located in the SFO (sfo) and choroid plexus (cp) in the areas of cerebral ventricle. B. A adjacent brain section incubated with sense APA ^{35}S-riboprobe. Note the lack of labeling of the SFO and CP (arrows). C. APA mRNA was also found in the PVN (pvn) and ME (me) of the brain. D. Control sections incubated with sense APA ^{35}S-riboprobe had no APA mRNA labeling in the area of the PVN and ME (arrows). Bar = 2 mm.

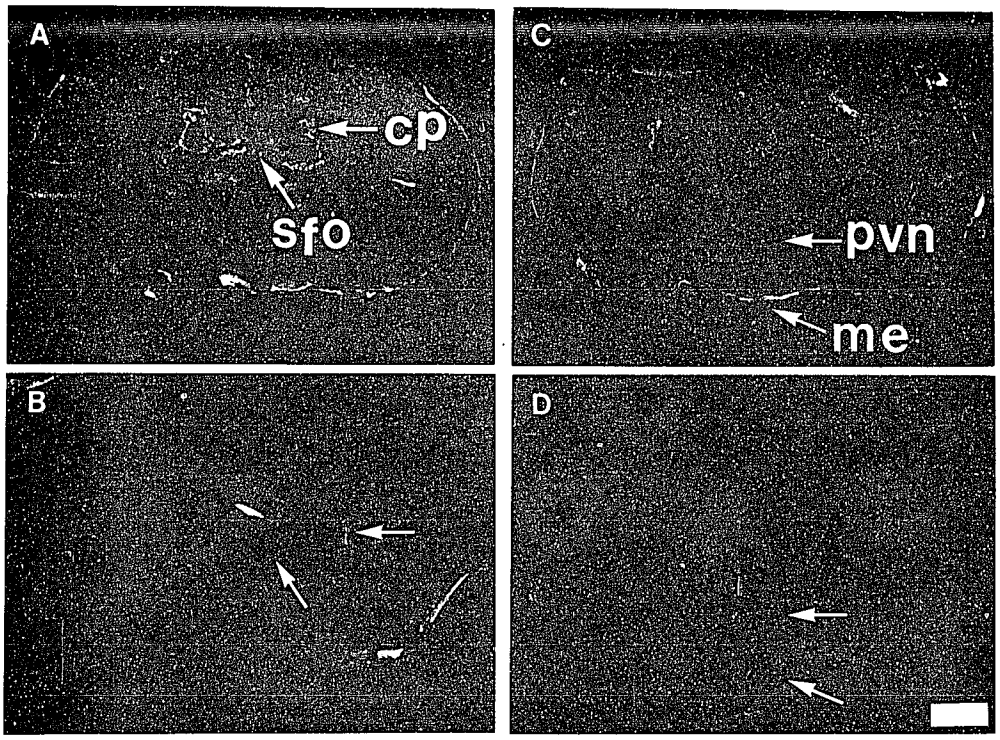


Figure 4

APA Activity in Cerebral Microvessels

	µmole/mg protein/h	Enrichment	n
Kidney homogenate	13.48 ± 1.00	-	10
Brain homogenate	0.21 ± 0.01	1	6
50 µm sieve material	2.23 ± 0.81	10.5	6
Sucrose density gradient			
1.2 M	1.81 ± 0.63	8.5	4
1.5 M	1.86 ± 0.50	8.7	5
1.7 M	4.83 ± 1.01	22.7	5

Table 1. APA activity in kidney, brain and cerebral microvessels isolated from rat brain by sieving and sucrose density gradient centrifugation. Enzyme activity was measured as µmole of substrate cleaved per mg protein per h. Enrichment refers to the amount of APA activity in the sieved and sucrose gradient material divided by the APA activity in the crude brain homogenate. Values are expressed as mean ± SEM. n equals the number of experiments.

Chapter 4

EFFECT OF AII INFUSION ON KIDNEY APA ACTIVITY

INTRODUCTION

AII is the active peptide of the renin-angiotensin system (RAS) in peripheral tissues (Catt et al., 1984; Goodfriend et al., 1977). The mechanism of feedback regulation of AII on its biosynthetic enzymes, including renin, the rate-limiting enzyme of AII synthesis, and angiotensin converting enzyme (ACE) is well known (Garrison and Peach, 1990). Relatively little is known about whether AII regulates the activity of the peptidases that degrade it. APA, a specific enzyme for converting AII into AIII (Kugler, 1982; Abhold and Harding, 1988), is generally believed to be an unregulated housekeeping enzyme. Wolf et al. (1990) reported that APA activity within glomeruli is reduced by ACE inhibitor treatment in uninephrectomized rats with partial kidney ablation, suggesting indirectly that glomerular APA is upregulated by the high levels of intrarenal AII in this model. Several studies indicate that chronic AII infusion in the rat is associated with the augmentation of intrarenal ACE and systemic angiotensinogen (Thun et al., 1994; Wolf et al., 1990). To further understand the functional relationship between AII and APA in the kidney, we studied the effect of subcutaneous AII infusion on renal APA. The experimental data demonstrate that AII increases APA expression in the kidney glomeruli.

MATERIALS/METHODS

Experimental design

Male Sprague-Dawley rats (175-225 g, Charles River Breeding Laboratories, Wilmington, MA) were housed on a 12 h light/12 h dark schedule and allowed free access to food and water.

Two groups of rats (6 rats/group) were left nephrectomized under pentobarbitol (50 mg/kg, i.p.) anesthesia. For the AII group, an osmotic minipump (Model 2001; Alza, Palo Alto, CA) containing synthetic AII (Sigma Chemical, St. Louis, MO.) was implanted subcutaneously at the dorsum of neck of left nephrectomized rats in order to deliver AII 2.4 ug/ul/hr. For the control group, a minipump was inserted at the dorsum of the neck of left nephrectomized rats to deliver sterile saline, 1 ul/hr.

Either one or two weeks post-surgery, the rats were sacrificed, blood serum collected and the right kidneys taken for RNA isolations, tissue sectioning, and isolation of kidney membrane and glomeruli.

The isolation of kidney glomeruli was conducted as previously described (chapter 2).

BP measurement

After 6 or 12 days post-surgery, blood pressures were measured by using a tail-cuff sphygmomanometer (IITC Inc.). The data were analyzed statistically by t-test.

APA Enzyme assays

APA enzyme activity from kidney membranes was measured by using alpha-glutamyl-2-naphthylamide (Bachem Bioscience, Philadelphia, PA) as substrate. The concentration of the protein was determined according to the method of Bio-Rad Protein Assay (Bio-Rad Laboratory, CA). Specific activities are expressed as U/mg protein, where 1 U equals the hydrolysis of 1 nmole substrate per hr (Wilk and Thurston, 1990).

Immunoblots

Immunoblots of kidney membranes (40 ug protein/sample) and isolated glomeruli (20 ug protein/sample) from AII infusion and control infusion groups were conducted as previously described (chapter 2) with some modifications. Briefly, for immunoblot of kidney membranes, after incubation with APAAb (1:1000 dilution) overnight, the membrane containing protein samples was washed with PBS, incubated either with peroxidase labeled secondary Ab and visualized by diaminobenzdine method as mentioned in immunoblot of chapter 2, or incubated with ^{125}I-protein A (4000,000 cpm/10 ml) and autoradiographed at -80^{o}C for several days. Immunoblots of isolated glomeruli, after incubation with APAAb (1:3000 dilution), were incubated with

peroxidase labeled secondary Ab and washed with PBS. The protein samples on the membrane were visualized by luminescence (ECL Western blotting, Amersham International plc). The labeling intensity of the bands was measured by densitometry and photographed.

Northern blot

Total RNA from kidney was isolated according to the method of Chomczynski and Sacchi (1987). Briefly, 100 mg tissue in 1 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol and 0.5% N-lauroylsarcosine) was homogenized with a polytron for 20 sec. The homogenate was then mixed with 0.1 ml of 2 M sodium acetate, pH 4, 1 ml water-saturated phenol, and 0.2 ml of 49:1 chloroform/isoamyl alcohol. The resulting mixture was centrifuged at 10,000 g at 4°C for 20 min. The upper aqueous phase containing total RNA was transferred and precipitated with 100% isopropanol at -20°C for 30 min and centrifuged at 10,000 g for 10 min. The RNA pellet was resuspended in 0.3 ml denaturing solution, precipitated with 0.3 ml of 100% isopropanol at -20°C for 30 min and centrifuged at 10,000 g for 10 min at 4°C. The RNA pellet was resuspended in 75% ethanol at RT for 10 min and precipitated by centrifugation at 10,000 g for 5 min. After drying the RNA pellet in a vacuum for 5 min, the pellet was redissolved in diethyl pyrocarbonate-treated 0.5% SDS water and stored at -80°C.

Northern blot hybridization was conducted according to the method of Sambrook et al. (1989). Briefly, total RNA (10 ug/sample) was separated on a denaturing

agarose/formaldehyde gel in a running buffer of 1 X MOPS at 80 volts, at RT for 3 hrs. The separated RNA was then transferred to a nitrocellulose membrane in a transfer buffer of 20 X SSC at RT overnight. After the membrane was baked at 80°C for 2 hrs, it was placed in 20 ml of prehybridization solution (25 mM KPO4, pH 7.4, 5 X SSC, 5 X Denhardt's solution, 50 ug/ml salmon sperm DNA, and 50% formamide) and prehybridized at 39°C for 4 hrs. After removing the prehybridization solution, a hybridization solution containing labeled probe (see below) at about 500,000 cpm/ ml, 25 mM KPO4, pH 7.4, 5 X SSC, 5 × Denhardt's solution, 50 ug/ml salmon sperm DNA, 50% formamide, 10% dextran sulfate was added and the membrane hybridized at 39°C overnight. The membrane was washed with 1 X SSC containing 0.1% SDS at RT 15 min X 2, then with 0.25 X SSC containing 0.1% SDS at RT 15 min X 2. Then the membrane was autoradiographed at -80°C for about 3 days.

High specific activity DNA probes of APA were prepared using random primers with an NEBlot Kit (New England Biolabs, Inc., Beverly, MA). Briefly, 50 ng of template cDNA of APA was dissolved in 30 ul nuclease free water and was denatured at 100°C for 5 min. The tube containing the DNA was centrifuged briefly in the cold and the following reagents added sequentially: 5 ul 10 × Labeling buffer including random octadeoxyribonucleotides, 6 ul dNTP mixture (2 ul of dATP, dTTP, and dGTP), 5 ul α ^{32}P dCTP (50 uCi), and 1 ul DNA Polymerase I- Klenow Fragment (5 units). The reaction was carried out at 37°C for an hr. and then terminated by adding 5 ul of 0.2 M EDTA (pH 8).

APA Histochemistry

The histochemical staining of APA activity in rat kidney sections was conducted as previously described (chapter 2). The APA substrate was H-Glu-4MBNA (0.4mg/10 ml).

APA Immunohistochemistry

The immunohistochemical staining of APA with fluorescein-labeled avidin in rat kidney was carried out according to methods previously described (chapter 2).

RESULTS

BP responses

The BP responses of both AII- (40 ng/min) and saline-treated rats were moderately increased 6 days post-surgery (Fig.1). The systolic BP of the AII infused group was sightly higher than that of saline group, but this difference was not statistically significant ($p > 0.05$). Likewise, the BP difference between groups in two-week post-surgery rats was not statistically significant.

Enzyme activity

APA enzyme activity in kidney membranes from both one-week and two-week AII infusion groups was higher than in control rats ($P < 0.001$) (Fig. 2). There was no difference in serum APA activity between both one-week and two-week groups (Fig.2). APA activities of isolated glomeruli from either one-week or two-week AII infused rats were increased more than two fold compared to control rats. There was a positive correlation between the specific enzyme activities of kidney APA with the BP of the same rats receiving chronic AII infusion (Fig.3).

Immunoblots

Immunoblots with APAAb on kidney membranes from both one-week AII and control rats produced the same labeling pattern (Fig.4). However, the intensity of the APA bands at Mr 136 kDa from AII infused rat was much stronger than that from the control rats. The smaller band at Mr 107 kDa, which was previously shown to be DPPIV (chapter 2), was also strongly labeled in the AII infused rats compared to that in the control. Isolated glomeruli further demonstrated the difference in APA labeling intensities between AII infusion and control rats (Fig.5). Densitometric analysis indicated that APA levels were increased five fold and three fold in one-week AII and two-week AII, respectively, compared to controls. Furthermore, the intensity of the APA band in the 2 week AII-infusion group was stronger than that in control, while the intensity of the DPPIV bands in both groups were almost the same based on equal amount of protein (20 ug/sample).

Northern blot analysis

Northern blot hybridization was conducted on total RNA of both AII infused and saline infused rats. There was no apparent difference between AII and saline groups (Fig.6).

APA histochemistry

Histochemical studies of kidney sections from both AII and saline two-week-infusion rats demonstrated that APA activity in the glomeruli of AII infused rats was higher than that in the glomeruli of saline rats (Fig.7). The renal tubule staining was similar in both AII and saline groups.

APA immunohistochemistry

The immunofluorescent staining intensity in the glomeruli of AII infused rats was significantly increased compared to that of the saline infused rats (Fig.8).

DISCUSSION

In order to investigate the functional relationship between AII and APA, a selective enzyme for AII metabolism (Kugler, 1982), the effect of AII infusion on the regulation of intrarenal APA was studied in uninephrectomized rats. There was no

significant increase in BP in AII-infusion group compared to control. However, there was a positive correlation between the elevation of BP and kidney APA activity of individual rat within the AII-infusion group. These results suggest that the impaired renal function by AII infusion and uninephrectomy may result in a high glomerular capillary hydraulic pressure followed by glomerular sclerosis in the contralateral kidney and renal hypertension (Simons et al., 1994). Glomerular hypertension is consistent with systemic hypertension. However, the same augmentation of BP in both the AII-infusion and control groups made it less likely that the difference in intrarenal APA levels between the two groups was influenced by the glomerular hypertension.

Chronic AII infusion resulted in an increase in APA specific enzyme activity. This increase in APA activity is likely to be due to an increase in the level of APA proteins which were shown to be elevated in immunoblots of kidney membranes. There was no detectable difference in serum APA levels between AII-infusion groups and controls. The significant difference in APA activity between AII infusion and control groups was further demonstrated in the kidney glomeruli with the fact that no detectable change in APA was found in the lumen of renal tubules of AII rats compared to the control. It appeared that the increase in APA level was primarily due to an increase in glomerular expression of APA. APA activity was increased in isolated glomeruli, as was APA histochemical activity in kidney sections. Glomerular APA immunocytochemistry was also increased. There was no difference in APA mRNA levels between the kidneys of the two groups as seen in Northern blots. However, since only a single time point was measured, it is not possible to determine whether the increase in APA levels is due to an increase in transcription, RNA stability or enzyme half life.

AII receptors are known to be concentrated on mesangial cells (Burns et al., 1993). Mesangial cell contraction stimulated by AII in the kidney glomeruli reduces capillary permeability, decreases glomerular filtration followed by the volume retention and renal hypertension (Burns et al., 1993). Chronic AII infusion is associated with the augmentation of both intrarenal and systemic AII as well as the elevation of intrarenal ACE (Thun et al., 1994). The significant elevation of intrarenal APA in the presence of high AII levels in the AII infused rat suggests that the intrarenal APA is under the regulation of AII locally. Consistent with this, there was a positive correlation between the elevations of blood pressures and the increases in the specific enzyme activities of glomerular APA in the AII-infusion rats. In addition to the changes of intrarenal RAS including ACE and APA as mentioned above, renal hypertension caused by chronic AII infusion is accompanied by the structural injury of the kidney with the proliferation of the smooth muscle cells of renal vasculature and the mesangial cells of renal glomeruli, and the fibrosis of renal tubulointerstitial tissues (Johnson et al., 1992; Burns et al., 1993). It is possible that the augmented APA levels within mesangial cells may protect against the hypertensive effects of AII by degrading AII locally, thereby reducing the contractile effect of AII on mesangial cells, reversing the increased hydrostatic pressure of glomeruli, and reducing the structural alterations of glomeruli. The upregulation of intrarenal APA may represent an example of a homeostatic defense mechanism to prevent the pathological development of AII related renal hypertension.

Therefore, the upregulation of intrarenal APA, especially in the glomerular mesangial cells, by AII may play an important role in preventing the development of

renal hypertension. However, the possible influence of the high glomerular hydrostatic pressure on the intrarenal RAS is not eliminated. The questions of AII regulation on the APA activity in the absence of glomerular hypertension still need to be studied.

Fig.1. Bar graph show systolic blood pressure in one-week (n = 9) and two-week (n = 6) AII infusion plus left nephrectomized rats, and in left nephrectomized control rats (n = 6). Where no significant differences present between the AII-infusion rats and control rats in either one-week or two-week groups.

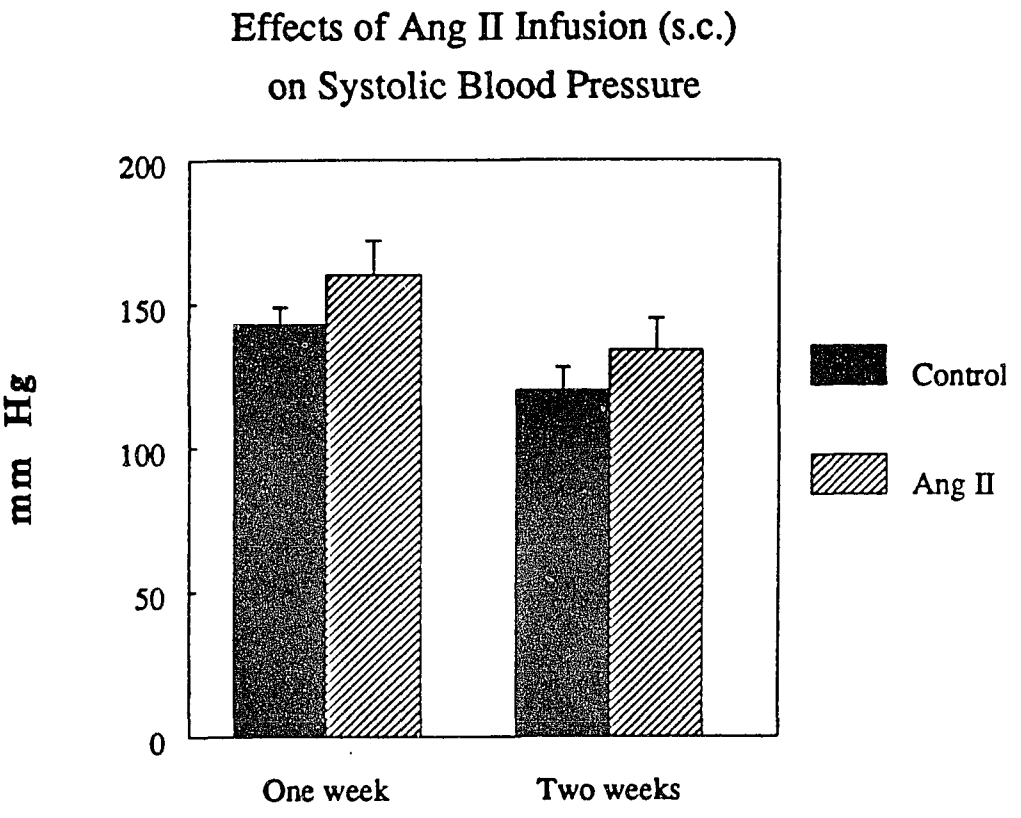

Effects of Ang II Infusion (s.c.)
on Systolic Blood Pressure
mm Hg
200
150
100
50
0
One week
Two weeks
Control
Ang II

Figure 1

Fig.2. Bar graphes show kidney APA activity in one-week (1 wk AII), two-week (2 wk AII) AII infusion plus uninephrectomy rats and uninephrectomy only (control) rats. The specific enzyme activity of APA is expressed as umol/mg protein/hr (tissue) or μmol/ml (serum). Data are mean ± SEM of six animals in each experimental group. In total kidney membrane, APA SEA of the AII infused rats (1 wk AII and 2 wk AII) is significantly higher than that of control rats, the data were analyzed by t-test with *P = 0.001 vs control. The SEA of APA in the isolated glomeruli of 1 wk AII is 2.4 fold higher than that of control, and of 2 wk AII is 2.1 fold higher over that of control. Serum SEA of APA are the same for all three rat groups as shown in the left columns of the figure.

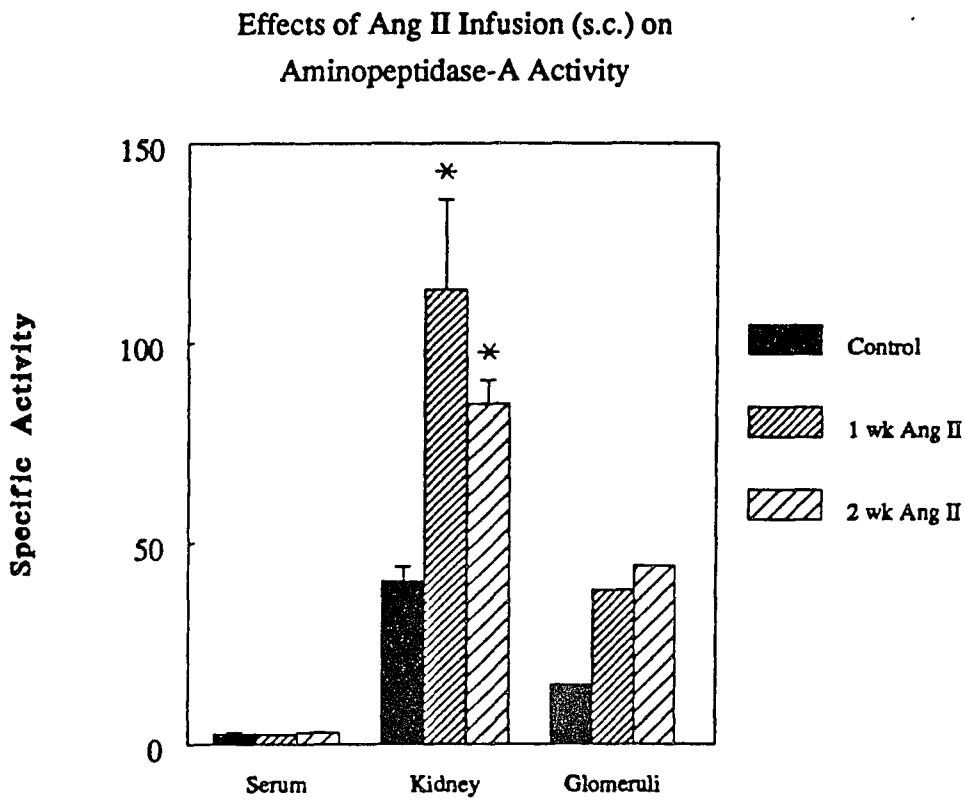

Effects of Ang II Infusion (s.c.) on
Aminopeptidase-A Activity
Specific Activity
150
100
50
0
*
*
Serum
Kidney
Glomeruli
Control
1 wk Ang II
2 wk Ang II

Figure 2

Fig.3. Linear regression analysis for the changes on BP and kidney APA activity in AII infused rats. Note that there is positive correlation between elevation of BP and APA enzyme activity. r = 0.72.

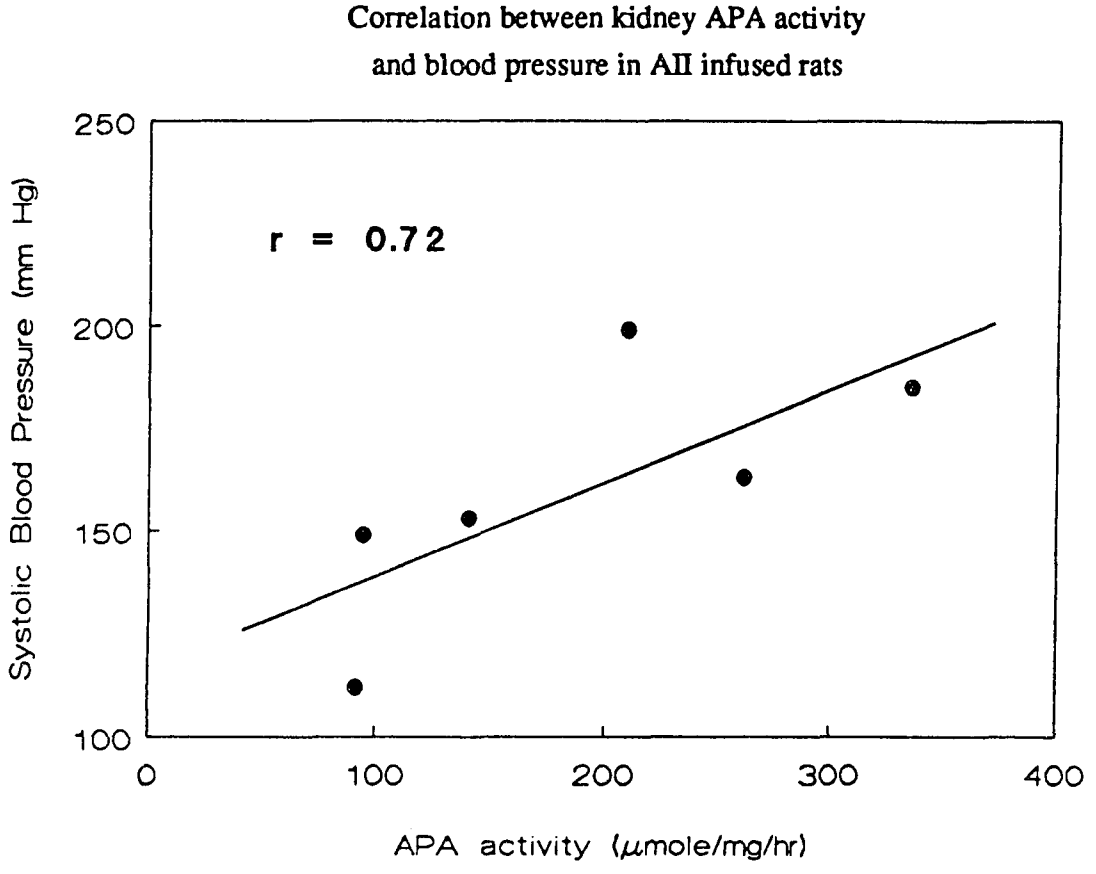

Correlation between kidney APA activity
and blood pressure in AII infused rats
r = 0.72
Systolic Blood Pressure (mm Hg)
250
200
150
100
0
100
200
300
400
APA activity (μmole/mg/hr)

Figure 3

Fig.4. APA immunolabeling with diaminobenzdine method in immunoblot of kidney membranes from one-week AII infusion plus uninephrectomized rats (n = 6) and one-week saline infusion uninephrectomized (control) rats (n = 6). The intensities of the APA bands M$_r$136 kDa from the AII infused rats was much stronger than that from the control rats. While the intensities of the smaller bands M$_r$106 kDa which representing the bands of DPPIV (see previous study in chapter 3) were also strongly labeled in the AII infused rats compared to that in the control.

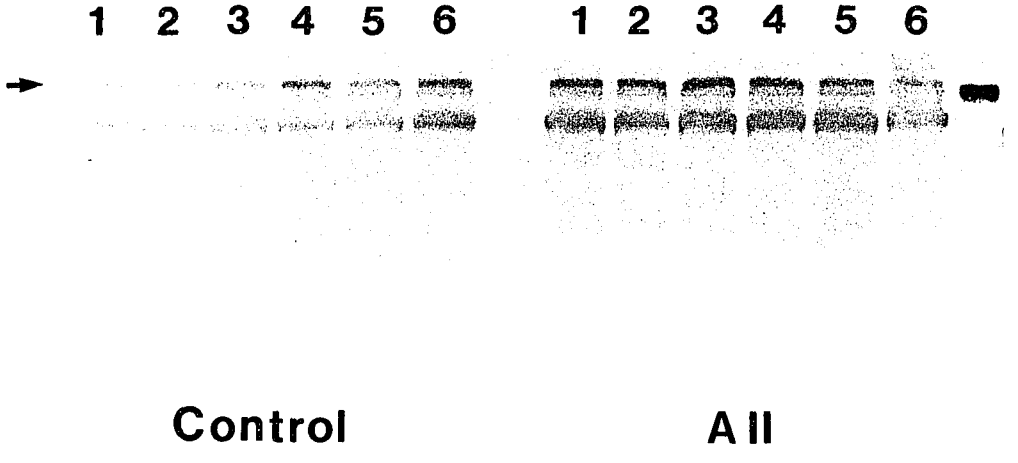


Figure 4

Fig.5. ECL Western Blotting of the isolated glomeruli from 1 wk AII and 2 wk AII rats plus uninephrectomy, and uninephrectomy only (control) rats. There are about four times stronger labeling intensities in the APA bands of 1 wk AII (lane 2) and 2 wk AII (lane 3) rats over that of control (lane 1) based on densitometric quantification. Note that the APA bands (arrow) of 2 weeks AII infusion rats are much stronger than that of control, while the DPPIV bands of both groups were almost the same.

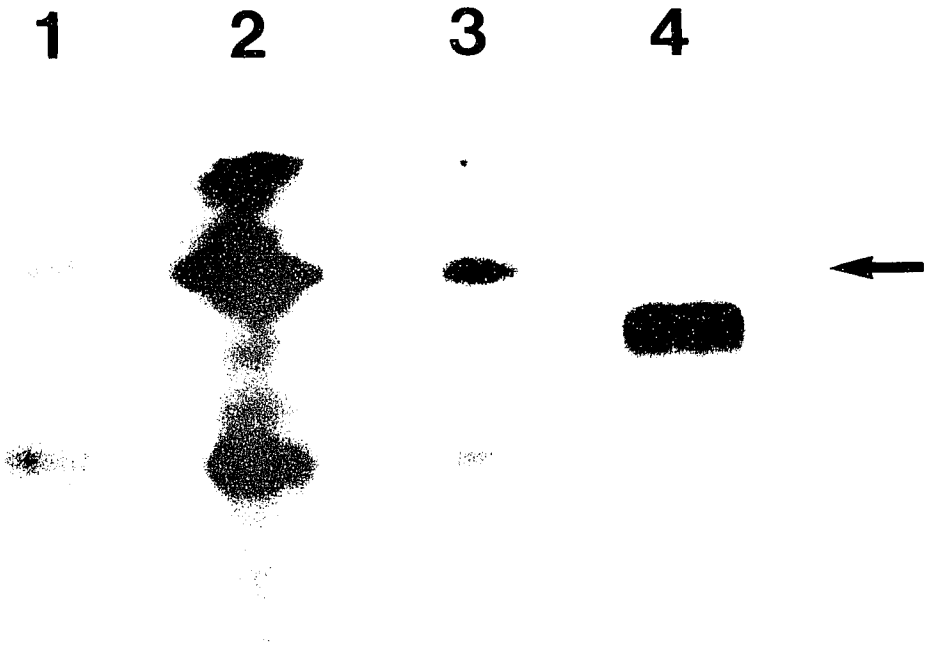


Figure 5

Fig.6. Autoradiography shows Northern Blot analysis of APA mRNA with total RNA isolated from both one-week AII infusion plus uninephrectomy rats (n = 6) and one-week saline infusion plus uninephrectomy rats (n = 6). 10 ug of RNA was analyzed in each sample. APA mRNA bands were labeled with the APA ^{32}P-DNA probe, where there is no significant difference between the AII group and control group.

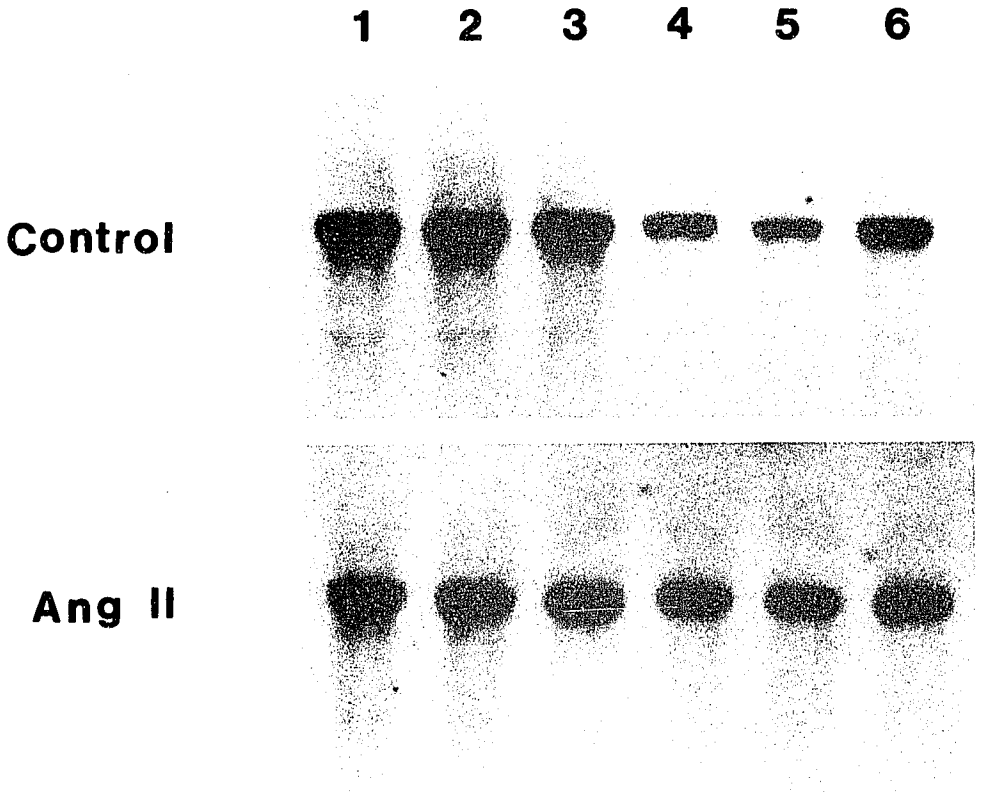


Figure 6

Fig.7. Histochemical studies of the sections from one week and two weeks AII infusion rats and control rats. Note that higher APA activities were found in the glomeruli of AII infused rat kidneys (1 week AII infusion: panel C. and 2 weeks AII infusion: panel E, arrows) compared to the glomeruli of control rat kidney (panel A. arrow). While the APA activities in the renal tubules of both rats are the same. Bar = 50 μm. This labeling difference was also demonstrated in the higher power views of the glomeruli from AII infusion (panel D. and panel F.) and control (panel B.) rats. Bar = 50 μm.

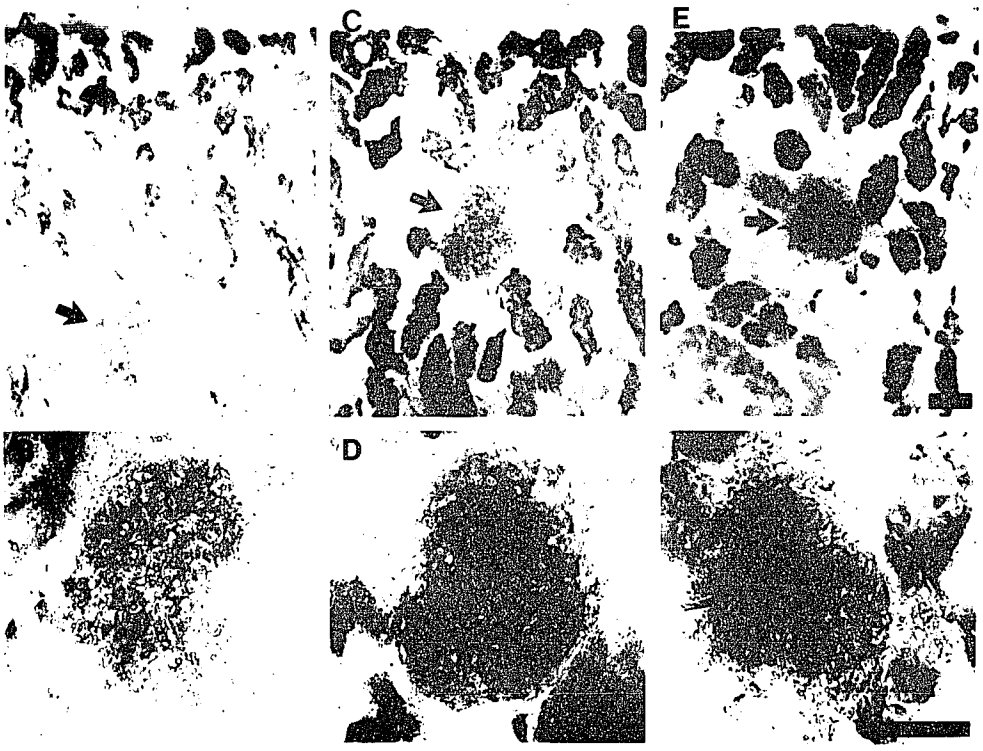


Figure 7

Fig.8. Immunofluorescent labeling of kidney sections of one-week AII infusion (panel B.), two-week AII infusion (panel C.) and control (pane A.) rats. APA immunofluorescent intensity is higher in the glomeruli of AII rats than that of control rats. There is no clear difference in labeling intensity of renal tubules between these two groups. Bar = 50 μm.

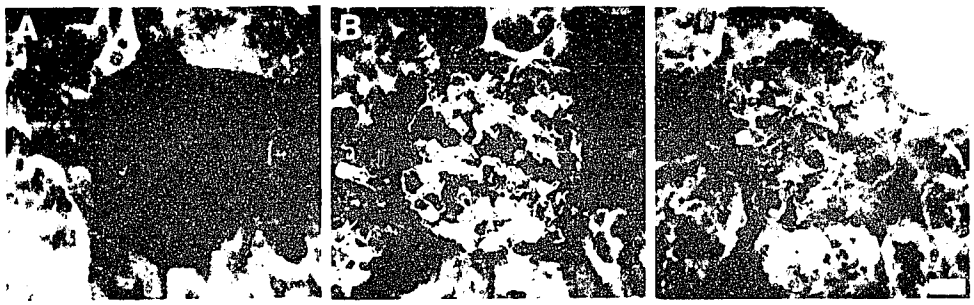


Figure 8

Chapter 5

CHARACTERIZATION OF APA IN 2K-1C RENAL HYPERTENSION RATS

INTRODUCTION

In the development of renal hypertension, renal artery stenosis related hypoperfusion is a major factor for the upregulation of renin secretion (Garrison and Peach, 1990; Burns et al., 1993). In the kidney, the regulation of renin secretion in response to renal hypoperfusion, systemic fluid and electrolyte imbalances, and the levels of the bioactive peptide AII are well known (Garrison and Peach, 1990; Antonipillai et al., 1989). High levels of AII stimulate renal hypertension by direct constriction of glomerular mesangial cells and blood vessels, stimulation of sodium reabsorption in renal proximal tubules, and positive feedback regulation of its own production by elevation of intrarenal ACE and systemic angiotensinogen levels (Wolf et al., 1990).

AII is the principal active angiotensin peptide of RAS in the peripheral tissues (Catt et al., 1984; Goodfriend et al., 1977). The metabolism of AII by APA, an enzyme that cleaves selectively the N-terminal Asp residue of AII to produce AIII, is suggested by several studies in both serum and brain of rat (Nagatsu et al., 1965; Abhold and Harding, 1988). AIII is then further degraded by other enzymes including aminopeptidase M (Kugler, 1982) to the inactive peptide fragments (Garrison and Peach, 1990). Early studies indicated that APA is an unregulated housekeeping enzyme (Nagatsu et al., 1965). A report by Wolf et al. (1990) suggests that AII may regulate APA activity based on the fact that glomerular

APA activity is reduced by ACE inhibitor treatment in uninephrectomized rats with partially ablated kidney. Previous studies on kidney APA activity in chronic AII infused rats (chapter 4) further demonstrated that high levels of AII upregulate glomerular APA activity. Within the AII infusion group there appeared to be a positive correlation between blood pressure and kidney APA activity. The influence of moderate hypertension in the chronic AII infused rats on intrarenal APA can therefore not be separated completely from a direct effect of AII. The problems imposed by the glomerular hypertension in chapter 4. can be resolved by using the Goldblatt hypertensive rat model. In the two-kidney, one clip (2K-1C) Goldblatt hypertensive rats, blood flow to one kidney is impaired by a silver clip resulting in a hypotension (Morishita et al., 1993). Animals develop an hypertension that is manifested around 4-6 weeks. The unclipped kidney is exposed to high AII and high pressure. The clipped kidney is exposed to high AII but low perfusion pressure. Thus the regulatory effect of AII on the kidney APA can be studied in the clipped kidney in the absence of blood pressure effects. The results indicate that APA was consistently increased in glomeruli from both clipped and unclipped kidneys, indicating that AII increases APA expression by a direct mechanism.

MATERIALS/METHODS

Experimental design

Two groups of male Sprague-Dawley rats (6 rats/group, 175-225 g, Charles River Breeding Laboratories, Wilmington, MA) were used for the experiment and were housed on a 12 h light/12 h dark schedule and allowed free access to food and water.

For the 2K-1C group, a silver clip (0.2 mm) was placed over the left renal artery of rats under pentobarbitol anesthesia (50 mg/kg, i.p.).

During the experimental procedure, the blood pressures of both groups of rats were measured once a week for four successive weeks by using tail-cuff sphygmomanometer (IITC Inc.). Four weeks post-surgery, the rats were sacrificed, the blood serums of all rats were collected, the left (clipped or normal) kidneys and the right kidneys of rats were taken for tissue sectioning, kidney membrane and glomeruli isolations.

The preparation of kidney membranes and isolation of kidney glomeruli from both 2K-1C group and control group was conducted as previously described (chapter 2).

Enzyme assays

Kidney membranes or isolated glomeruli were homogenized with a polytron for 20 sec. in 0.05 M TrisHCL buffer, pH 7.5. APA enzyme activities were measured as described previously (chapter 2).

Immunoblot

Immunoblot for the proteins of isolated glomeruli from both groups of rats was conducted according to the methods described previously (chapter 4). The method of ECL Western blot with APAAb (1:3000 dilution) was used here. The labeling intensity of the bands was measured by densitometry.

Histochemistry

The histochemical studies of the sections from clipped kidney and unclipped kidney of 2K-1C rats, and control kidney after surgery for four weeks were conducted the same way as that described in chapter 2.

Immunohistochemistry

Immunofluorescent labeling of kidney sections of both 2K-1C rat kidneys and control rat kidney was carried out according to the methods described in chapter 2.

RESULTS

BP responses

BP increased progressively in the 2K-1C and control rats through the 4 weeks of the study (Fig.1). Both the systolic BP and the mean BP of 2K-1C rats were slightly higher than that of control rats; this difference was statistically significant ($P < 0.05$) in the three-week and four-week post-surgery rats .

Enzyme assays

APA enzymatic activity of total rat kidney membranes was higher in control rats compared to that in both clipped and unclipped kidneys of 2K-1C rats ($P < 0.05$) (Fig.2).

There was no difference in APA activities in serum from both clipped rats and control rats. However, APA enzyme activity within the isolated glomeruli of unclipped kidney of 2K-1C rats was increased about 1.7 fold over that of the normal kidney. Strong fibrosis of the clipped kidney interfered with the purification of isolated glomeruli and prevented measurement of glomerular APA activity. However, histochemical studies indicated that APA activity in the glomeruli from clipped kidney was actually higher than from unclipped and control kidneys (see below).

Immunoblot

Immunoblots of isolated glomeruli from clipped, unclipped, and normal kidneys revealed that the APA labeling intensity of glomeruli from the unclipped kidney was about twice as strong as that of control glomeruli (Fig.3., Fig.4). APA labeling from clipped kidney glomeruli was less compared to glomeruli from the unclipped one. Because of the difficulty in obtaining isolated glomeruli from the clipped kidney, a direct comparison of APA levels cannot be made. However, in glomeruli from the clipped kidney, the intensity of the APA band was much stronger than the APM or DPPIV bands compared to the relative staining of the three bands from control glomeruli (Fig.3). Thus, APA levels also appear to be elevated in the clipped kidney glomeruli.

Histochemistry

Histochemical studies of sections from unclipped and clipped kidneys indicate that APA activities in glomeruli were markedly elevated compared to control glomeruli (Fig.5).

The clipped kidney glomeruli exhibited some fibrosis and glomeruli were considerably smaller than glomeruli from the unclipped kidney.

Immunohistochemistry

Sections of unclipped and clipped kidneys showed significant increases in APA immunofluorescence in the glomeruli compared to that of control rats (Fig.6). Consistent with the histochemistry observations above, most tubulointerstitial tissues in the clipped kidney were replaced by fibrosis, and the size of glomeruli of the clipped left-kidney was much smaller than that of normal kidney. The latter was also smaller than that of unclipped kidney. However, the tubular elements from the unclipped kidney appeared normal. The intensity of APA immunofluorescent labeling in the proximal tubules of unclipped kidney was the same as that of normal kidney.

DISCUSSION

Previous studies (chapter 4) indicated that chronic AII infusion upregulated glomerular APA activity. However, since blood pressure was moderately elevated in these animals, the possible influence of moderate hypertension on the glomerular perfusion pressure and the kidney APA levels could not be eliminated. The Goldblatt renal hypertensive rat model (2K-1C) has been studied for a long time (Ploth, 1983; Okamura et al., 1986). Clipping of the left renal artery results in the development of renal hypertension due to : 1. Increased renin release from the clipped kidney regulated by hypoperfusion (Schricker et al., 1994; Garrison and Peach, 1990). 2. Increased intrarenal

ACE of both kidneys in spite of the suppressed renin levels in the unclipped kidney by AII stimulation (Thun et al., 1994). 3. Increased levels of systemic AII as well as clipped kidney AII (Morishita et al., 1993). 4. Increased injures of the kidney mediated by either direct AII stimulation of the glomeruli and tubulointerstitial tissues or hypoperfusion of the clipped kidney. Therefore, the Goldblatt hypertensive (2K-1C) rat is a very good model for studying the AII regulation on APA in the kidney.

The BP of 3 to 4-week post-surgery rats with 2K-1C was significantly higher than that with control rats, consistent with continuous secretion of renin from the clipped kidney and increased AII levels in the systemic circulation in spite of the physiological feedback inhibition of renin secretion by AII. APA enzymatic activity was higher in kidney membranes from control rats compared to that of both clipped and unclipped kidneys of 2K-1C rats ($P < 0.05$). However, higher resolution histochemical and immunohistochemical methods indicated that APA was increased in the glomeruli of both kidneys of 2K-1C rats. While there was an equal intensity of APA staining presenting in renal tubules from both unclipped and control kidneys, there was almost no APA staining in the tubulointerstitial tissues of the clipped kidney. The high APA activity of kidney glomeruli in the 2K-1C rats compared to control was confirmed in the isolated glomeruli by SEA of APA and immunoblots. The possible reasons for the results with kidney membranes are: 1. Most of the tubulointerstitial tissues of left clipped kidney were replaced by fibrosis in presence of extremely high AII levels and hypoperfusion of the kidney, so that the normally large proportion of APA in renal tubules was diminished. Even though there were increased APA proteins in the glomeruli, the total SEA of APA in the clipped kidney was lower than that in the normal kidney. 2. There was a dramatic proliferation in the right unclipped kidney

stimulated by the very high levels of AII. The size of unclipped kidney (1.5 - 2 g) from 2K-1C rats was about two times larger than kidneys (1 g) from control rats, and the latter were twice the size of the clipped kidney (0.5 g). Consistent with this, studies of the kidney in chronic AII infusion rat by Johnson et al. (1992) indicate that a high level of α-smooth muscle actin is found in the glomeruli and the renal interstitium, there was a proliferation of desmin proteins in the glomeruli and smooth muscle cells in the blood vessels, and the injured tubulointerstitial cells also have marked proliferation. Thus, because of the increase in kidney connective tissue, the SEA of APA per kidney in 2K-1C rats would be reduced compared to that of the normal kidney even though APA level is actually increased. In the unclipped kidney, the glomerular perfusion pressure as well as the ACE and AII should be high because of the renal hypertension associated with the augmented circulating AII of 2K-1C rat (Ploth, 1983). Thus the increased glomerular APA in the unclipped kidney may be the results of AII stimulation from either systemic circulation or elevated intrarenal RAS in response to glomerular hypertension.

High levels of circulating AII in the 2K-1C rats could result in a sustained vasoconstriction and injury to the glomerular microcirculation. Anoxia of the glomerular tissue caused by both AII stimulation and hypoperfusion may result in focal and segmental glomerular sclerosis as seen in the clipped kidney with smaller glomeruli compared to control glomeruli. The pathophysiological consequences of glomerular sclerosis could be impaired renal fluid homeostasis and body fluid retention, while the dilution of sodium concentration after body fluid overload could further stimulate the augmented intrarenal RAS and make the renal hypertension worse. Therefore, there may be a vicious cycle developed between the renal hypertension and the elevation of intrarenal RAS in 2K-1C

rats, especially in the clipped kidney. Furthermore, the increased renin secretion of clipped kidney was strong enough to override the suppressive effect of augmented AII levels in the kidney and systemic circulation (Garrison and Peach, 1990). Therefore, the elevation of glomerular APA of clipped kidney was the direct stimulation of augmented AII from either systemic circulation or increased intrarenal renin.

Studies by Nagatsu et al. (1965) indicated that APA was a unregulated housekeeping enzyme for there was no detected elevation of serum APA activity in hypertensive or renal hypertensive patients. Therefore, the lack of elevation in APA activity in the serum of 2K-1C rat is consistent with the human studies. Based on the studies in both serum and kidney APA activity, it is suggested that APA is regulated by AII locally in the tissues. The source of serum APA is not clear yet, thus it is possible that the serum APA activity may be influenced not only by circulating AII levels but also by unclear sources.

Therefore, these experiments demonstrated that the upregulation of intrarenal APA by AII was at the protein level qualitatively and quantitatively. APA, a specific metabolizing enzyme for AII, is regulated by its substrate AII in the kidney. The pharmacological benefits of ACE inhibitor to block the synthesis of AII for the treatment of renal hypertension have been known for about 3 decades (Garrison and Peach, 1990). The pharmacological significance of APA in the peripheral metabolism of AII and in the regulation of AII stimulated renal hypertension is suggested here.

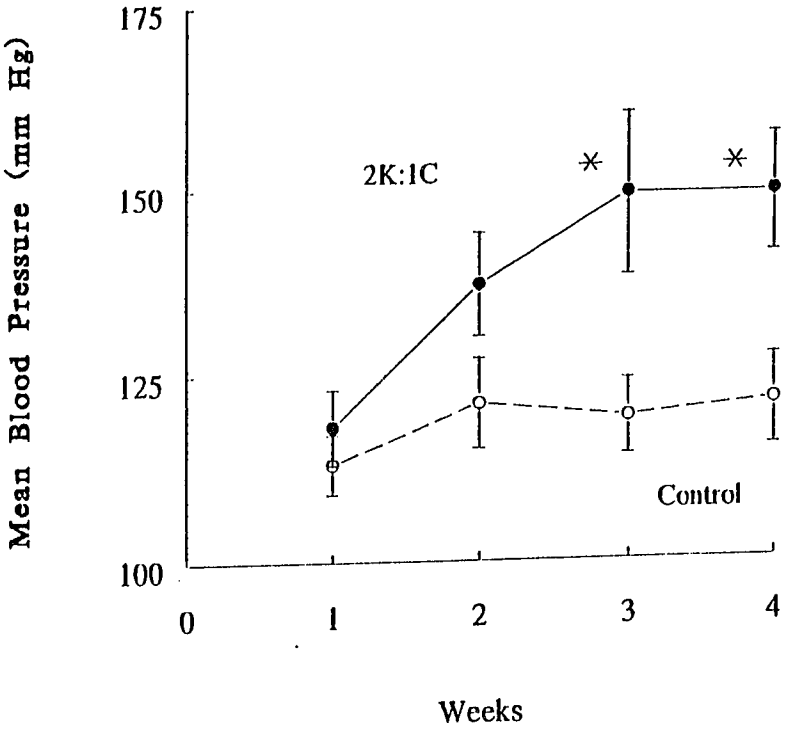


Fig.1. Mean blood pressure in left renal artery clip (2K-1C) rats and normal control rats. Symbols are: (● - ●) 2K-1C (n = 18); (o - o) Control (n = 8); except in the four week rats of 2K-1C: n = 17, Control: n = 3. *P < 0.05 vs Control.

Fig.2. Bar graph of kidney APA activity in left renal artery clip (2K-1C) rats and normal control rats. The specific enzyme activity of APA is expressed as umol/mg protein/hr. Data are mean ± SEM of six animals in each experimental group. In the cortex, APA SEA of the control is significantly higher than that of 2K-1C, *$P < 0.05$ vs control. The SEA of APA in the isolated glomeruli of unclipped kidney of 2K-1C rats is 1.7 fold higher over that of control. There is no data shown here for the glomeruli of clipped kidney which was unsuccessfully isolated due to the fibrosis of the kidney as mentioned in the text.

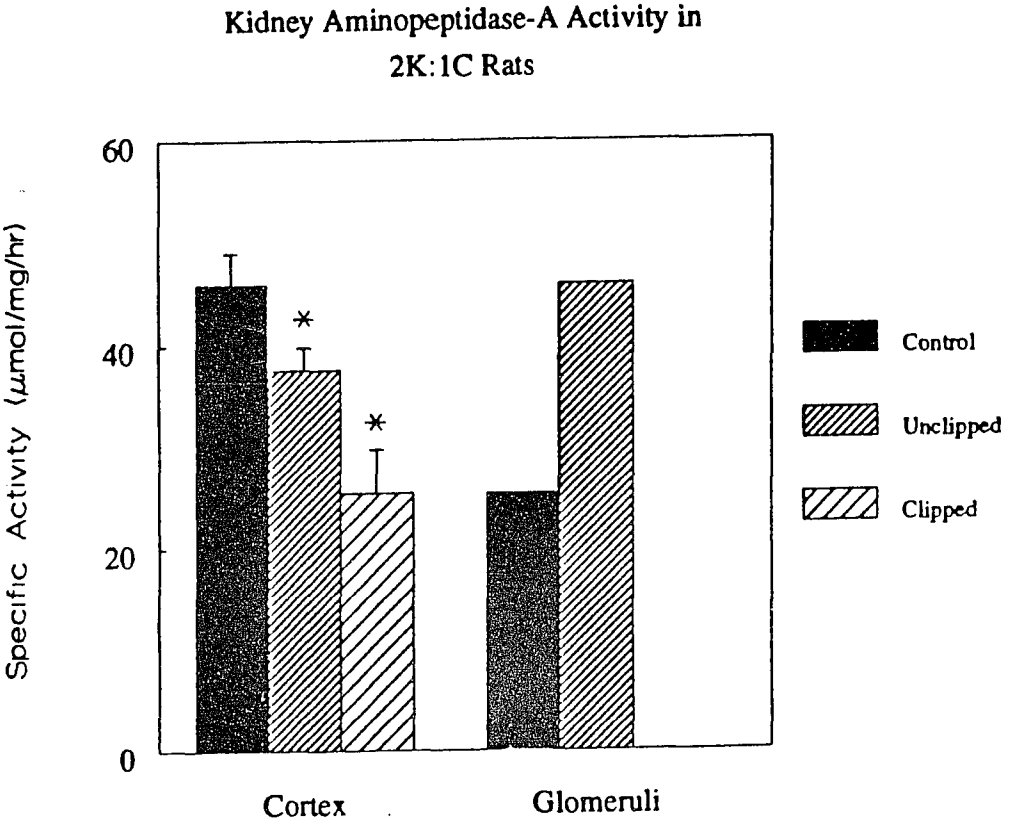

Kidney Aminopeptidase-A Activity in
2K:1C Rats
Specific Activity (μmol/mg/hr)
60
40
20
0
*
*
Cortex
Glomeruli
Control
Unclipped
Clipped

Figure 2

Fig.3. Western blotting of the isolated glomeruli from the unclipped (lane 2), clipped (lane 3) kidneys of 2K-1C rats and control (lane 1) rats. APAAb was at 1:3000 dilution. Note that APA immunostaining is much stronger in the bands of unclipped glomeruli compared to that of control, while clipped glomeruli is not as strong as that of unclipped one due to the unsuccessful isolation procedures mentioned in the text.

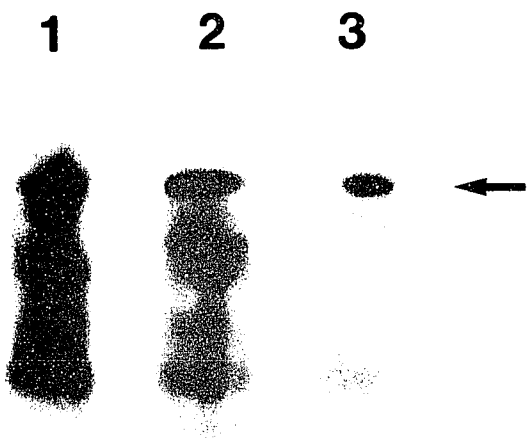


Figure 3

Fig.4. Western blot with isolated glomeruli of unclipped kidney and control kidney. Lane 1. Control glomeruli. Lane 2. Unclipped glomeruli. Note that APA band (arrow) of the unclipped glomeruli is much stronger than that of control glomeruli.

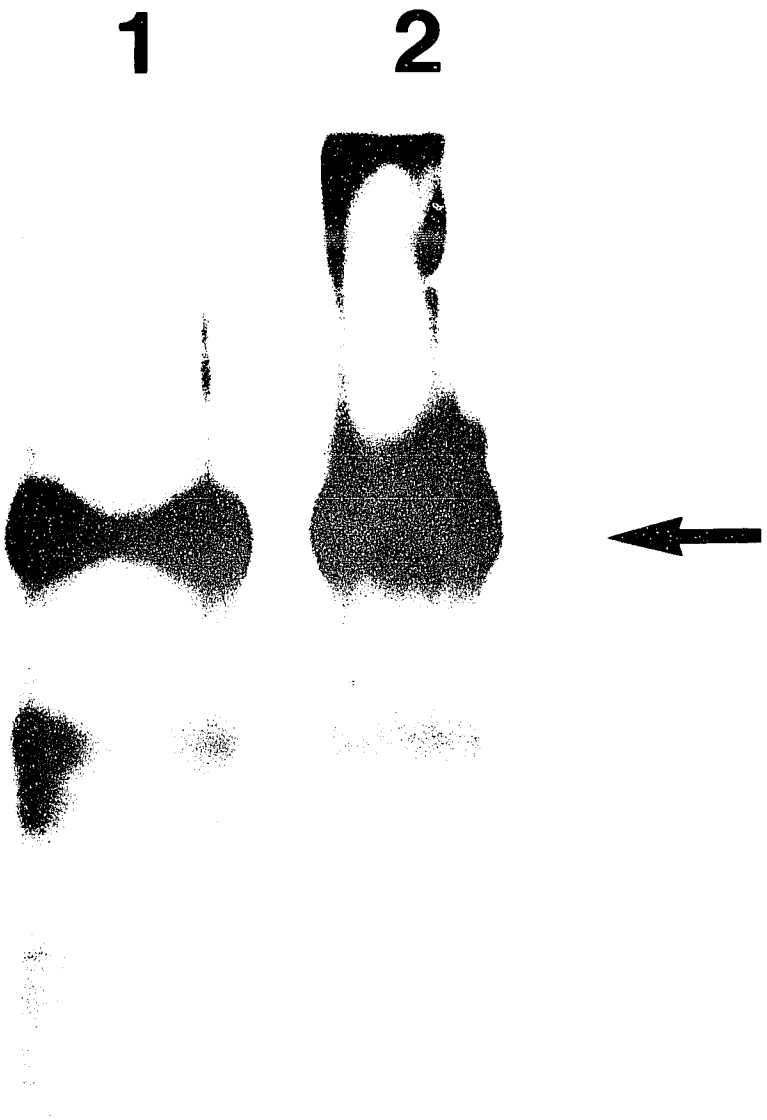


Figure 4

Fig.5. Histochemical studies of the sections from 2K-1C rats and control rats. At low power, the glomeruli of both unclipped (C. arrow) and clipped (E. arrow) kidneys of 2K-1C rats had much higher APA activities compared to control glomeruli (A. arrow). The glomeruli of clipped kidney were much smaller in size than the glomeruli of both control and unclipped kidneys, and the unclipped glomeruli were also larger than the control glomeruli. There was intensive tubulointerstitial fibrosis in the clipped kidney (E.). Bar = 50 mm. In a high power view of the glomeruli of the three tissues (B.D.F.). Note the APA activity was higher in both clipped (F.) and unclipped (D.) glomeruli of 2K-1C rats compared to the control (B.). Likewise the size of unclipped glomerulus was larger than control glomerulus, and the later was larger than clipped glomerulus. Bar = 50 mm.

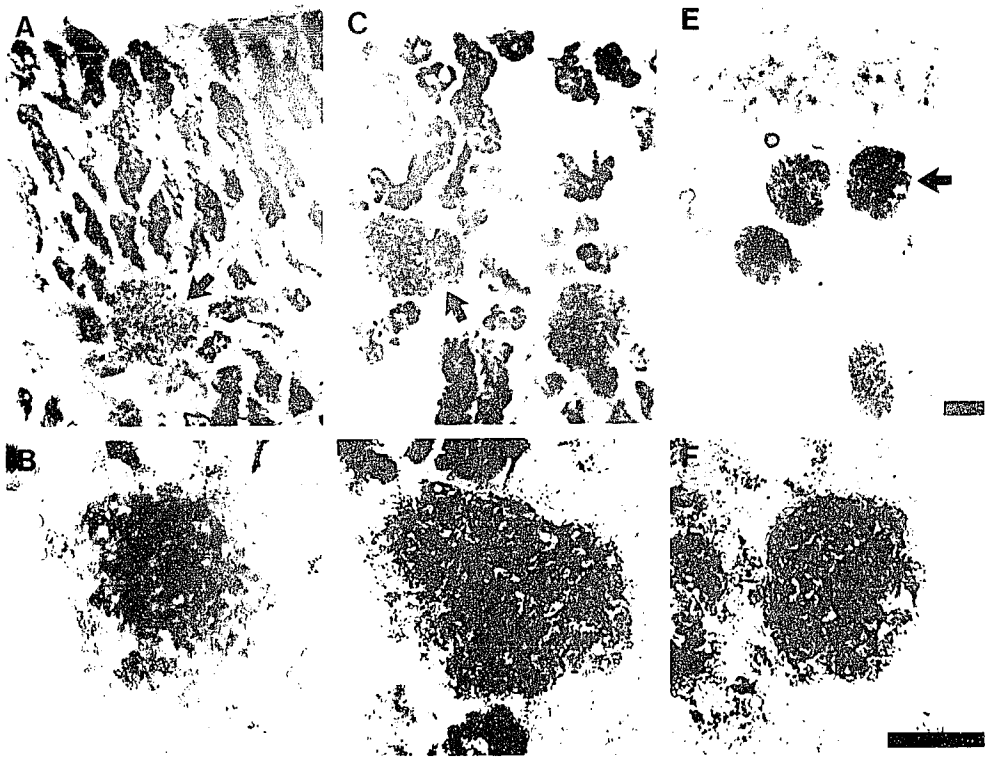


Figure 5

Fig.6. APA immunofluorescent labeling of both unclipped and clipped kidneys of 2K-1C rats compared to control kidney. A. In the normal kidney of control rats, glomeruli had very faint APA immunofluorescent labeling (arrow). B. The APA immunofluorescent labeling was clearly present in the glomeruli of unclipped kidney (arrow). C. There was intense APA immunofluorescent labeling in the glomerulus of clipped kidney (arrow). D. Autofluorescence in a section without antibody. Note the APA labeling intensity of both glomeruli from clipped (C.) and unclipped (B.) kidneys was stronger than the normal glomerulus from control (A.) kidney. Likewise the size of the clipped kidney glomerulus (C.) was much smaller than the control glomerulus (A.), and the later was smaller than the unclipped kidney glomerulus (B.). Bar = 50 μm.

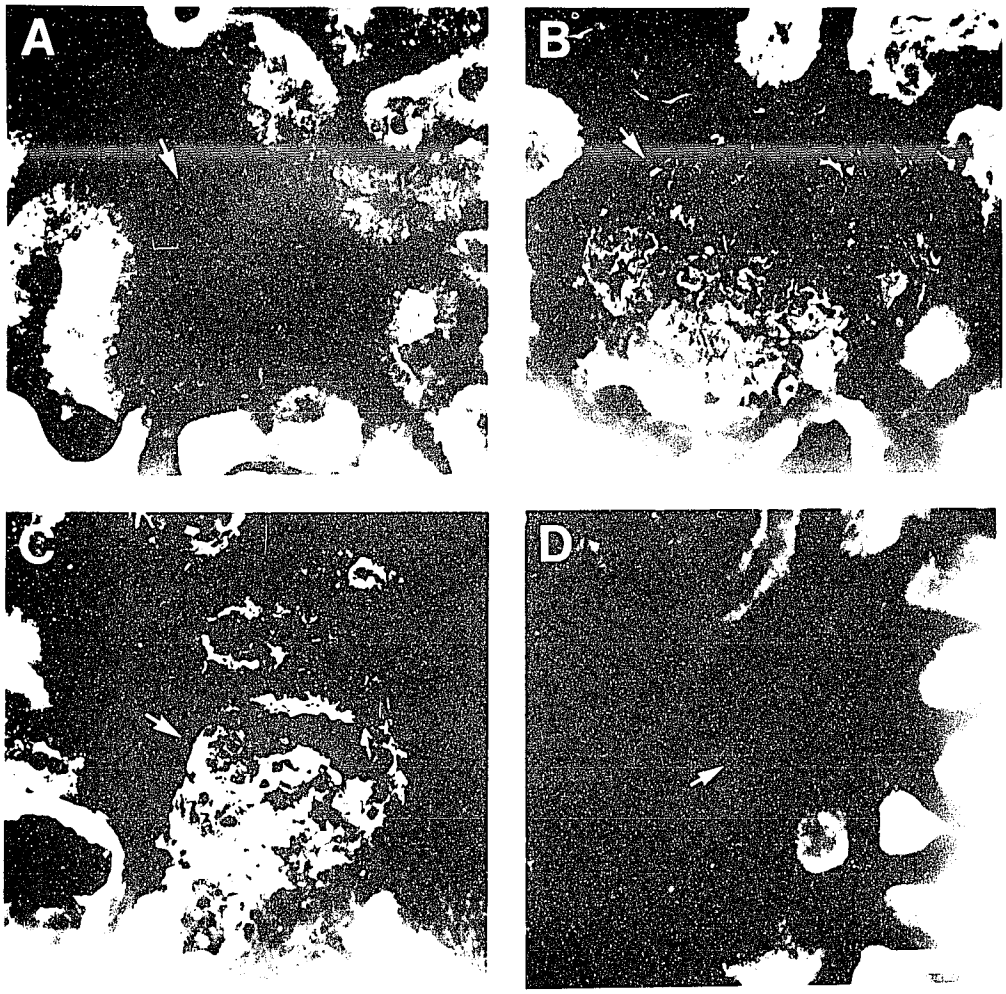


Figure 6

Chapter 6

DISCUSSION

1. THE BIOLOGICAL EFFECTS OF ANGIOTENSIN PEPTIDES AND AII METABOLISM

The importance of endocrine and paracrine functions of renin-angiotensin system (RAS) in regulating blood pressure and fluid volume has been well established. RAS-related hypertension is the result of AII stimulation on peripheral tissues and AII or AIII effects on the brain. AII binds to the AT_1 receptor site, a G-protein coupled receptor, to activate phospholipase C (Sumners et al., 1991) and exert its hypertensive effects on many tissues (Garrison and Peach, 1990). AII is known to exert its effects on both the structure and tone of the vasculature. In addition to being a potent vasoconstrictor, AII stimulated hypertension depends on AII increased sympathoexcitation by increasing the central sympathetic outflow and the release of catecholamines from the adrenal medulla (Fitzsimons, 1980; Peach, 1986); and on AII stimulated fluid volume retention by increasing the water drinking response of SFO in the brain, the synthesis of vasopressin from periventricular nucleus (PVN), and the synthesis as well as release of aldosterone from adrenal cortex (Bell et al., 1984; Fitzsimons, 1980). AII is also a trophic factor for stimulating collagen synthesis in vascular smooth muscle cells in vitro (Kato et al., 1991).

Chronic hypertension is associated with arterial wall hypertrophy and increased collagen content (Hajdu et al., 1991). The medial thickness of cerebral vessels in

spontaneously hypertensive rats is reduced by angiotensin converting enzyme (ACE) inhibition (Clozel et al., 1989). ACE inhibitors not only block the synthesis of AII but also block the degradation of bradykinin, a potent vasodilator (Garrison and Peach, 1990; Rocha et al., 1949). Thus it has been known for a long time that ACE inhibitor treats effectively many kinds of human hypertensive diseases stimulated by AII (Antonaccio, 1982; Williams, 1988), especially for the treatment of renovascular hypertension (Williams, 1988; Garavaglia et al., 1988) and congestive heart disease (Pfeffer et al., 1988).

In the synthetic pathway of the RAS, it is known that the synthesis and release of renin from kidney is regulated by three factors: renal perfusion, renal sodium load, and β_1-receptor stimulation. By converting angiotensinogen to AI, renin is the rate-limiting enzyme of the biosynthetic pathway of RAS. ACE converts AI to the octapeptide AII by cleaving the Phe^8-His^9 bond of AI (Garrison and Peach, 1990). To block the synthesis of AII, a lot of effort has been focused on the inhibition of ACE in both biological research and clinical pharmacology (Clozel et al., 1989). However relatively less is known about the pharmacological significance of AII catabolism. Although it was believed that many angiotensinases including aminopeptidase A (APA) are involved in degrading AII (Garrison and Peach, 1990; Kugler, 1982), studies by Abhold and Harding (1988) indicate that the first step in AII metabolism is by APA to cleave specifically the N-terminal Asp residue of AII to produce AIII. AIII is then degraded into inactive fragments by other peptidases including aminopeptidase M (APM) cleaving the Arg^2-Val^3 bond, prolyl endopeptidase cleaving the Pro^7-Phe^8 bond, and dipeptidyl aminopeptidase III (DPPIII) cleaving the Val^3-Tyr^4 bond (Abhold and Harding, 1988., Garrison and Peach, 1990).

APA is a membrane-bound and glycosylated ectoenzyme (Kugler, 1982). The identification of the amino acid sequence of APA indicates that it is one of the metallopeptidase enzyme family with a conserved zinc-binding domain (Wu et al., 1990). APA is 30% similar in amino acid sequence to APM, also a membrane-bound enzyme (Kugler, 1982).

Much evidence indicates that local RASs exist. AII and AII receptors are located in a variety of tissues including many areas of brain, different parts of kidney, cell membranes of anterior pituitary and cardiomyocytes, and sinusoids of the adrenal cortex (Mendelsohn, 1984; Unger et al., 1988; Moffett et al., 1987). AII is the potent angiotensin peptide in peripheral tissues. The $t_{1/2}$ of AII (4.4 min in blood, 23 sec. in CSF) is longer than that of AIII (2 min in blood, 7.7 sec. in CSF) (Gaynes et al., 1978; Harding et al., 1986). Therefore, the rapid degradation of AIII indicates that AIII may be more potent than previously considered (Ahmad and Ward, 1990; Nagatsu et al., 1965). Indeed, inhibition of APM resulted in the equipotent stimulation of BP by AIII compared to AII in the periphery (Ahmad and Ward, 1990). Likewise AII and AIII show similar binding affinity to the AII receptor in the presence of the APM inhibitor amastatin (Fujimoto et al., 1992), suggesting that the rapid degradation of AIII may make it less potent. AIII has also been suggested to be equal in potency or more potent than AII in the central stimulation of water drinking and pressor reaction (Moffett et al., 1987; Felix and Schlegel, 1978; Harding and Felix, 1987; Wright and Harding, 1992). With the suggested paracrine effect of AII in different tissues, the potent hypertensive effect of AIII in the brain (Okamura et al., 1992; Lilly et al., 1985; Campbell, 1987; Stier, 1989; Felix and Schlegel, 1978), and the specific functions of APA for the metabolism of AII (Abhold and Harding, 1988), the

functional relationship between AII and APA in the development of hypertension in different tissues especially in the brain and kidney becomes important.

AII is know to regulate its synthesis by feedback inhibition of renin secretion (Garrison and Peach, 1990). However, relatively little is known about AII regulation of its own degradation. Likewise, the regulatory effect of AII on APA is not clear. APA is believed to be a unregulated housekeeping enzyme based on the fact that there is normal serum levels of APA in hypertensive patients (Nagatsu et al., 1965), and APA as a membrane-bond ectoenzyme, with the active site facing the extracellular fluid. In contrast, renin secreted by the JG cells of kidney is under the regulation of many factors, including AII. A recent study by Wolf et al. (1990) indicates that the elevated APA activity from uninephrectomized rats with partially ablated kidneys is reduced by ACE inhibitor treatment, suggesting that APA is upregulated by the high levels of AII in this model. Therefore, the study of the regulatory effect of AII on APA will help in further understanding the metabolism of AII and the importance of APA in regulating the endocrine and paracrine effects of AII on the development of hypertension.

2. APA IN THE PERIPHERAL TISSUES

Local RASs are found in many tissues as shown by biochemical and molecular biological studies. Angiotensinogen and renin mRNA are found in rat tissues such as brain, cardiomyocytes and vasculature (Campbell, 1987; Okamura et al., 1992; Lilly et al., 1985; Dostal et al., 1992). AII receptors are localized in adrenal cortex and medulla, in the cell membranes of cardiomyocytes, anterior pituitary and hepatocytes (Mendelsohn, 1985). AII

is generally believed to be the active peptide in the peripheral RAS (Catt et al., 1984; Goodfriend et al., 1977). APA, a membrane-bound ectoenzyme involved specifically in degrading AII to AIII (Abhold and Harding, 1988), is abundant in the renal tubules and glomeruli of rat kidney indicated by histochemical studies (Kugler, 1982). Based on the broad tissue distribution of the RAS and the paracrine effect of AII on a variety of tissues, studies on the cellular localization of APA in different tissues of rat would help us to further understand the importance of APA in regulating the effect of AII locally. With the newly developed rabbit antiserum against purified APA (APAAb) of rat kidney, and the cloning of partial cDNA of APA from rat kidney, APA activity and APA mRNA were characterized in different rat tissues by immunologic and in situ hybridization techniques.

Immunoblots of kidney homogenate, kidney glomeruli and outer medulla with or without preabsorption of purified APM or DPPIV (Chapter 2) indicated that APAAb cross-reacts with APM and DPPIV of rat kidney. Thus the major band M_r 136 kDa is APA, the band M_r 129 kDa is APM and the band M_r 107 kDa is DPPIV. The smaller single band of purified APA of rat kidney may be the truncated form of APA due to the process of purification described previously (chapter 2). However, at the higher dilutions used for immunocytochemistry, there was little apparent cross-reactivity of the APA antiserum against APM or DPPIV (Chapter 2).

Immunocytochemistry produced strong APA staining of renal proximal tubules. The physiological functions of APA in the lumen of proximal tubules may be related to indirect regulation on AII stimulated reabsorption of Na and H_2O, and mediation of the reabsorption of amino acids as a general peptidase together with other peptidase to degrade different

peptides. The co-localization of the AII receptor (Mendelsohn, 1984) and APA in the JG cells (Chapter 2) suggests that APA may regulate indirectly renin release by regulating AII levels locally. Glomeruli were also positively stained by immunocytochemistry, but staining was sensitive to the type of fixation that was used (Chapter 2). Immunofluorescent staining was consistent with labeling of mesangial cells. High levels of APA mRNA and proteins were demonstrated in the kidney glomeruli (Chapter 2), especially in the glomerular mesangial cells which contract in response to AII stimulation (Mendelsohn, 1984); suggesting the importance of APA in the kidney glomeruli for modulating the effect of both circulating and locally formed AII on the glomerular filtration. Consistently, both pericytes of cerebral microvessels and mesangial cells of kidney glomeruli are perivascular adventitial cells, derived from the same progenitor cells (Sims, 1986); both cells are the major sources of APA in rat brain and kidney (Chapter 2; Chapter 3). Therefore, APA in JG cells, the lumen of renal proximal tubules, and the glomerular mesangial cells may play an important role in regulating the effects of AII on intrarenal renin release and renal fluid homeostasis.

AII and AIII are equipotent in stimulating zona glomerulosa of the adrenal cortex to increase the synthesis and release of aldosterone. AII also stimulates cortisol secretion from zona fasciculata cells and catecholamine release from adrenal medulla. APA was determined to be associated with sinusoids of the three zones of adrenal cortex (Chapter 2). This localization suggests that APA is involved in converting AII to bioactive peptide AIII for aldosterone secretion from the zona glomerulosa and regulating AII effects on the production and secretion of other adrenal hormones of cortisol and epinephrine.

Along the hypothalamus-pituitary axis, the sinusoids of the hypophyseal portal system connect the capillaries of the median eminence to the cells of the anterior pituitary. Circulating or locally produced AII stimulates the release of prolactin and adrenocoticortrophic hormone (ACTH) from the anterior pituitary (Fitzsimons, 1980). AII and AII receptors (Mendelsohn, 1985) and APA (Chapter 2) are all present in the anterior pituitary. Thus APA associated with the sinusoids of anterior pituitary may provide an immediate regulation of the endocrine/paracrine effect of AII on the endocrine functions of pituitary hormones. AII also stimulates ADH release from the posterior pituitary. The localization of APA in the capillary of posterior pituitary (Chapter 2) suggests that APA may regulate the levels of AII locally around the extracellular fluid environment and within the blood vessels.

The liver exhibits a number of responses to AII stimulation including increased glycogenolysis and gluconeogenesis, inhibition of fatty acid synthesis, and stimulation of angiotensinogen production (Campanile et al., 1982). The localization of APA in the hepatocyte membranes (Chapter 2) indicates that APA may indirectly modulate AII effects on the physiological functions of liver.

Less is known about the physiological function of AII in the intestine. AII receptors are not found in the lumen of intestine. The high levels of APA mRNA and proteins on the columnar cells of intestine (Chapter 2) suggest its active role as a general peptidase for the absorption of dietary amino acids.

In the circulatory system, the enriched capillaries in the lung alveoli are responsible for the physiological exchange of O_2 and CO_2 of the body with the environment. AII has a weak vasoconstrictor effect on the lung (Garrison and Peach, 1990). AII receptors are associated with endothelial cells and smooth muscle cells of the blood vessels (Catt et al., 1984; Grammas et al., 1989). ACE is located in the endothelial cells of the lung (Stalcup et al., 1982). ACE activity is about 10 times higher in the lung than other tissues (Sattar et al., 1985). The conversion of AII generated by ACE into AIII in the pulmonary circulation is very low with almost no loss of AII in the pulmonary vascular bed of isolated perfused lung (Bakhle et al., 1969). Our studies indicated that APA was located in capillaries of the lung, but the localization to the endothelial versus the adventitial surface could not be determined. However, the low levels of AIII in the pulmonary circulation would suggest that APA is located within lung capillary pericytes consistent with localization to cerebral microvessel pericytes and mesangial cells within the kidney. On the adventitial side of blood vessels, APA would not be able to contact directly with AII formed on the endothelial cell surface by ACE, but would protect the lung from the stimulation of circulating AII that leaks into the interstitial space.

Therefore, the broad tissue distribution of APA is consistent with the endocrine and paracrine functions of AII in these tissues. Especially for the peripheral regulation of blood pressure, APA may be important in regulating indirectly AII effects on ADH release from the pituitary, aldosterone release from the adrenal cortex, catecholamine release from adrenal medulla, and renal fluid homeostasis.

3. FUNCTIONAL RELATIONSHIP BETWEEN APA AND AII IN THE BRAIN

The brain contains all the components of the RAS (Campbell, 1987; Wright and Harding, 1992). AII and AIII may act as neurotransmitters or neuromodulators to elevate blood pressure (Moffett et al., 1987), stimulate drinking, and release pituitary hormones. Immunocytochemical and electrophysiological studies of the rat brain RAS (Wright and Harding, 1992; Fitzsimons, 1980; Ganong, 1984; Rettig et al., 1986; Brattstrom, 1992; Reid, 1992) indicate that the PVN may act as an integration center, processing the incoming information of circumventricular organs (CVO), sending signals to the posterior pituitary (PP) for releasing ADH and to the nucleus tractus solitary (NTS) of the brainstem, the baroreflex center. AII, AII receptors and APA are abundant in the PVN, CVO , choroid plexus, ME, posterior pituitary and NTS (Chapter 2 and 3). APA in the brain is primarily associated with the adventitium of cerebral microvessels where pericytes are located (Healy and Wilk, 1993). AIII is a potent angiotensin peptide for central stimulation of water drinking and pressor effect (Felix and Schlegel, 1978; Harding and Felix, 1987) and it has been reported that AII and AIII are equipotent in the brain (Wright and Harding, 1992). Therefore it is suggested that AII and/or AIII stimulated elevation of blood pressure are dependant on both ADH release via CVO-PVN-PP pathway and sympathoexcitation via CVO-PVN-NTS pathway in the brain.

Immunoblots of rat kidney and cerebral microvessels with APAAb resulted in a similar labeling pattern (Chapter 3), suggesting that APA associated with the cerebral microvessels could also cleave the N-terminal aspartyl or glutamyl residues of oligopeptides including AII (Abhold and Harding, 1988). Interestingly, among the bioactive peptides

associated with cerebral vasculature (Uddman and Edvinsson, 1989), AII is the only known peptide containing an N-terminal acidic amino acid, suggesting that APA is highly selective for the metabolism of AII in cerebral microvessels. APA immunostaining in the adventitium of cerebral microvessels suggests a possible localization of pericytes (Healy and Wilk, 1993). The pericyte is an adventitial cell located outside the capillaries and small arteries (Sims, 1986), and is a component of the blood brain barrier (BBB) (Risau et al., 1990). The localization of APA to pericytes was confirmed by immunocytochemical studies of rat brain (Chapter 3).

Therefore APA enriched in the cerebral microvessels pericytes and the choroid plexus of cerebral ventricle (Chapter 3) suggests that APA may play an important role in the central regulation of blood pressure by converting circulatory and locally produced AII to AIII in cerebrospinal fluid (CSF), and by activating or inactivating circulating and locally produced AII as a part of the BBB in each area of the nuclei along the two angiotensinergic pathways. On the other hand, APA as a part of the BBB may also play a generalized role to degrade peptides containing N-terminal acidic residues generated by other peptidases and to limit the accessibility of other circulating peptides to the brain interstitium. APA in the choroid plexus of cerebral ventricles may directly mediate the effect of AII on the volume of CSF (Saavedra, 1992).

4. FUNCTIONAL REGULATION OF APA BY AII IN THE KIDNEY

Recent studies indicate that all the components of RAS are present in the kidney (Burns et al., 1993; Wolf et al., 1990; Thun et al., 1994). Chronic AII infusion in the rat

is associated with the elevation of intrarenal ACE and liver angiotensinogen (Thun et al., 1994), although there is a suppressed expression of intrarenal renin gene by high levels of AII. AII is a potent angiotensin peptide in the peripheral tissues. APA, a specific enzyme for converting AII into AIII (Abhold and Harding, 1988), is generally believed to be one of the angiotensinases involved in metabolism of AII (Garrison and Peach, 1990) and is further believed to be an unregulated housekeeping enzyme (Garrison and Peach, 1990; Nagatsu et al., 1965). Recent studies from Wolf et al. (1990) on glomerular APA from partially ablated kidneys in uninephrectomized rats demonstrated that treatment with an ACE inhibitor reduced APA enzyme activity, suggesting that glomerular APA is upregulated by the high levels of AII in this model. To further understand the functional relationship between AII and APA in the kidney, we studied the effect of subcutaneous AII infusion on renal APA activity. Our results demonstrated that AII increases APA expression in kidney glomeruli.

High levels of AII from chronic AII infusion stimulated not only an elevation in APA proteins but also an increased specific enzyme activity of APA in the kidney glomeruli (Chapter 4), suggesting that the elevation of glomerular APA activity is mainly due to the increased APA expression in the kidney glomeruli. Taken alone, these results suggest that the high levels of AII upregulate its degrading peptidase APA in the kidney glomeruli. The hypertension developed in the AII infused rats was not significantly higher than that in the control rats. However, there was a positive correlation between the elevations of blood pressure and of APA activity in the AII infused rats (Chapter 4). The moderate hypertension developed in both AII and saline infused rats is accompanied by glomerular hypertension which could result in renal proliferations in the vasculature,

glomerular mesangial cells, and tubulointerstitial tissues (Johnson et al., 1992), and produce a pressure natriuresis with a reduction in the fractional excretion of sodium in the glomeruli (Mark and Kline, 1994). This impaired renal function followed by renal hypertension may produce further augmentation of the intrarenal RAS which again could cause further renal impairment (Simons et al., 1994; Johnson et al., 1992). Therefore the possible influence of glomerular hypertension on the level of intrarenal APA is not eliminated in the chronically AII infused rat.

To address the question as to wether the change in APA levels was due to a direct effect of AII or an indirect effect of arterial pressure (glomerular pressure), we utilized the two-kidney one-clip (2K-1C) Goldblatt hypertensive rats. Clipping of one renal artery produces hypoperfusion of the clipped kidney (Morishita et al., 1993) and increased renin release from the clipped kidney (Garrison and Peach, 1990), elevating AII locally and systemically. The AII effect on APA levels in the clipped kidney (high AII, low pressure) can then be compared to the unclipped kidneys (high AII, high pressure).

APA expression was significantly increased in the glomeruli of both kidneys of the 2K-1C rat compared to that of the control rat (Chapter 5). It was noted in the experiment that the hypertension in the 3-4 week 2K-1C rats was significantly higher than in the control rats (Chapter 5). However, the specific enzyme activity of APA in the kidney membranes of 2K-1C rats was even lower than that of control rats (Chapter 5). There were dramatic reduction in tubular APA of clipped kidney. The normal renal tubules in the clipped kidney were replaced by massive tubulointerstitial fibrosis, the size of the glomeruli as well as the whole clipped kidney was smaller than that of control, suggesting that the

reduced APA activity in the renal tubules may be the result of either destruction of the enzyme or downregulation of the enzyme activity due to the decreased glomerular filtration of AII to the renal proximal tubules. While the intensity of APA staining in the tubules of both unclipped and control kidneys were the same, the size of unclipped kidney was twice as big as the control kidney. Thus it is suggested that the reductions in the APA activity per total kidney protein may be the result of the increased non-APA proteins such as fibrinogen in the clipped kidney and smooth muscle actin in the proliferated unclipped kidney of 2K-1C rat under the stimulation of AII and hypoperfusion (Chapter 5; Johnson, 1992). Thus, it is suggested that intrarenal APA is regulated directly by its substrate AII.

Relatively little is known about the regulation of peptidases by their substrates. The hypothalamus-pituitary-end organ axis involved in the regulation of endocrine functions of a variety of hormones such as thyroid hormone, cortical steroid hormone, and luteinizing hormone are well known. Within this axis, only one study by Vargas et al. (1994) indicates that a peptide hormone regulates the activity of its degrading peptidase; thyrotropin releasing hormone downregulates its degrading peptidase - pyroglutamyl peptidase II in the adenohypophyseal cells of the anterior pituitary. Other peptidases, including endopeptidase 24.15 for the degradation of luteinizing hormone release hormone (LHRH) released from the hypothalamus (Lasdun et al., 1989), are not yet known to be regulated by their substrate peptides. Our results show that the intrarenal APA is regulated by its substrate AII and provide unique evidence of peptide regulation of its degrading peptidase in peripheral tissues. AII is the potent angiotensin peptide in peripheral tissues with a longer $t_{1/2}$ in the blood than that of AIII which is rapidly degraded (Gaynes et al., 1978; Harding et al., 1986; Abhold and Harding, 1988). Thus the metabolism of AII by APA to produce

AIII may be the rate-limiting step in the degradation of angiotensin peptides in the peripheral RAS.

Therefore, APA expression appears to be upregulated by AII within glomeruli under conditions where AII is elevated. The rapid metabolism of AII by APA to AIII in the kidney glomeruli may be possible for the low levels of AII locally and results in a series of beneficial consequences: reducing the effects of AII on mesangial cells; reversing the increased hydrostatic pressure of glomeruli; reducing the structural alterations of glomeruli. Thereby, conversely, it is concievable that low levels of APA may be involved in AII dependent human hypertension. Importantly, it appears that AII as the bioactive peptide of peripheral tissue RAS not only regulates its synthetic enzymes, renin and ACE, but also regulates its degrading enzyme APA in the kidney (Chapter 6). This may be the first report of a peptide hormone system regulating its own metabolism in peripheral tissues.

In conclusion, these studies suggest that APA is an important enzyme for regulating both the endocrine and paracrine effects of AII throughout the body. In the brain and adrenal, AII metabolism by APA may be a conversion step into the bioactive peptide AIII. In cerebral microvessel pericytes, APA as a part of the BBB may play an important role in the central regulation of BP. In the pituitary, kidney and adrenal, the conversion of AII by APA to AIII may be the rate-limiting step of AII metabolism. APA indirectly regulates BP and body fluid volume by affecting AII stimulated secretion of ADH from pituitary and aldosterone from adrenal, and renal fluid homeostasis. AII directly regulates its degrading peptidase APA in the rat kidney. The upregulation of APA by AII may play an important role in preventing AII dependent human hypertension.

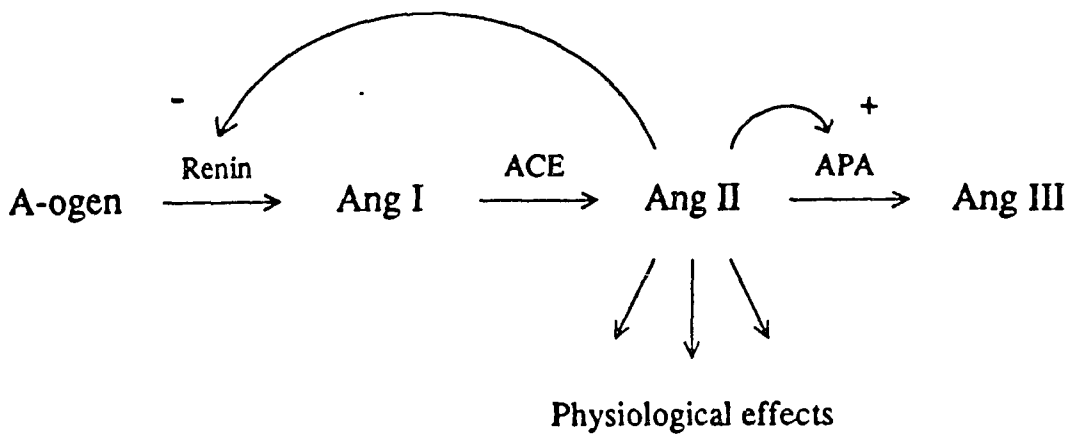

-
+
Renin
ACE
APA
A-ogen
Ang I
Ang II
Ang III
Physiological effects

Figure 1

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