Immunohistochemical Evidence that Angiotensins I and II Are Formed by Intracellular Mechanism in Juxtaglomerular Cells

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SUMMARY

The existence of angiotensin II (AII) immunoreactivity in juxtaglomerular (JG) cells of rat kidney, which has been demonstrated previously by immunohistochemical studies, can be explained either as the product of intracellular synthesis or by the internalization of receptor-bound AII originating in plasma. To resolve these two alternative mechanisms, attempts were made to identify AII in JG cells of rat kidney by immunohistochemical staining using specific antibodies to AII. Although AII-like immunoreactivity was not detected in normal rat kidney, rats treated with the angiotensin-converting enzyme inhibitors, MK-421 or captopril, showed AII-like immunoreactivity in JG cells. The presence of renin and AII-like immunoreactivity was demonstrated in the same cells by specific antibodies to respective antigens used on adjacent serial sections. These findings support an intracellular mechanism of the formation of AII and suggest an intracellular renin angiotensin system, presumably separate from the extracellular system.

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KEY WORDS

• renin • captopril • MK-421 • converting-enzyme inhibitor • immunohistochemistry • intracellular formation of angiotensin II • intracellular renin-angiotensin system

It was postulated that "intrarenal" angiotensin II (AII) might control several renal functions. Although "intrarenal AII" implied AII generated in the renal parenchyma, concrete experimental evidence was not available for such intracellular AII distinct from the peptide generated in renal circulation by the well-known extracellular mechanism.

Recently, Mendelsohn has reported the presence of AII in the extract of the unflushed kidney of the rat. We have observed coexistence of AII-like immunoreactivity and renin in rat juxtaglomerular (JG) cells by immunohistochemical studies providing evidence for the possible intracellular storage of AII. If AII in JG cells performs control functions, elucidation of the mechanism of its formation, accumulation, and release is of great importance for the evaluation of its functional significance in the kidney.

Two alternative mechanisms can be considered for the accumulation of AII in rat renal JG cells: 1) intracellular synthesis; or 2) accumulation of plasma AII, presumably through the internalization of the AII-receptor complex.

If AII is synthesized by an intracellular mechanism, AII should be present in the same cells, whereas if AII is taken up from plasma, it is unlikely that AII is accumulated by a possible internalization mechanism involving AII receptors of JG cells. With the objectives of identifying the mechanism of accumulation of AII in JG cells, we attempted to identify AII in JG cells by an immunohistochemical method. To arrest rapid conversion of AII to AII, JG cells of rats treated with the converting enzyme inhibitor (CEI) captopril or MK-421 were compared with those of untreated animals.

Materials and Methods

Treatment of Rats

Male Sprague-Dawley rats weighing approximately 250 to 300 g were treated with the CEI, MK-421 (Merck, Sharp and Dome Company) or captopril (Squibb and Sons, Inc.), for 15 days. MK-421 was administered intraperitoneally twice a day at a total daily dose of 5 mg/kg. Captopril was given orally twice a day at a total daily dose of 100 mg/kg. In addition, the inhibitors were also added to drinking water in a concentration of MK-421 of 4.2 mg/100 ml for rats treated with this drug, or of captopril 30 mg/100 ml. Animals were allowed free access to these solutions. The average intake of the drinking water was approximately 35 ml/rat/day, and there was no significant difference among MK-421-treated, captopril-treated, or nontreated rats in the daily intake.

On Days 8 and 15, 1 hour after the last intraperitoneal or oral administration of the CEI, rats were anesthetized with diabutal, perfused with 200 ml phosphate-buffered saline (PBS) containing 10 mM N-ethylmaleimide, 8 mM EDTA, 0.01 mM pepstatin, and 33 mM leupeptin followed by 250 ml Bouin's fixative. PBS without these inhibitors was also used in...
other experiments. Kidneys were cut into 4 mm thick slabs, postfixed in the same fixative for 2 hours, dehydrated, and embedded in paraffin.

Antisera

All antisera used in this study were raised in rabbits by the method of Vaitukaitis et al. Anti-renin antisera were produced with pure rat renal renin conjugated to tetanus toxoid. These antisera did not crossreact with human renin or rat cathepsin.

Antisera against Al and All were produced with conjugates of Al or All (obtained from Beckman) to bovine serum albumin or bovine thyroglobulin. In radioimmunoassay, anti-Al antisera showed less than 0.01% crossreaction with All, and anti-All antisera showed less than 0.5% crossreaction with Al.

Immunohistochemical Staining

Serial sections (4-5 μm thick) were mounted on glass slides coated with gelatin chromateum, and stained immunohistochemically using the unlabeled antibody peroxidase-antiperoxidase (PAP) method.* Primary antisera and control sera were diluted with PBS containing the mixture of inhibitors used for the perfusion of rats. Sections were incubated initially with these primary antisera solutions for 16 to 24 hours at 4°C, then with goat anti-rabbit IgG for 1 hour at room temperature, and finally with rabbit PAP complex for 1 hour at room temperature. Between each incubation step, the sections were washed three times with PBS. The immunoreactive substances were stained using diaminobenzidine and H₂O₂ as substrate of the peroxidase.

Control slides were treated similarly by using either normal rabbit serum or adsorbed antisera, which had been prepared by precubination with large excesses of the respective antigens for 48 hours at 4°C.

Results

Kidneys obtained from untreated rats showed strong immunostaining in JG cells with antirenin antisera (fig. 1 a) and anti-Al antisera (fig. 1 c), but no immunostaining was seen with anti-All antisera (fig. 1). Kidneys from rats treated with MK-421 or captopril for 1 week showed results practically indistinguishable from those of untreated rats (table 1). On the other hand, kidneys of rats treated with MK-421 for 2 weeks showed Al-like immunoreactivity (fig. 2 b) as well as renin (fig. 2 a) and All-like (fig. 2 c) immunoreactivities in JG cells. Likewise, kidneys of rats treated with captopril for 2 weeks showed positive immunostaining with antirenin, anti-Al, and anti-All antisera. No immunostaining was detected when primary antisera were substituted with normal rabbit serum or control antisera adsorbed with respective antigens either in the treated or untreated rats. Protease inhibitors were added to the perfusate (PBS) to prevent peptidolytic destruction of Al. The inhibitors dramatically improved the detection of Al-like immunoreactivity by increasing the number of Al immunoreactive JG apparatus by a factor of at least 3, and by increasing the intensity of staining. Use of the inhibitors did not affect the number of renin- or All-immunoreactive JG apparatus.

To obtain a quantitative relationship of cells containing renin, and angiotensin-like immunoreactivity, immunopositive JG apparatus in serial adjacent sections stained with antibodies to renin, Al and All, respectively, were counted (table 1). Of 400-600 renin-positive JG apparatus, approximately 50% were stained positively by Al antibody. CEI-treatment seemed to have little effect on this percentage. Of the renin-positive JG apparatus, approximately 14% became Al-positive by captopril administration for 2 weeks and 16% with MK-421. Conversely, whenever Al-like immunoreactivity was observed in a JG apparatus, it was also stained positively with antibody to renin. No general effect of CEI was observed on the length of the region of cells in afferent arterioles in which immunopositive cells were distributed. The difference in the length of immunopositive regions apparent in figures 1 and 2 seems to be merely coincidental. In general, the length seems to be quite variable from glomerulus to glomerulus.

Discussion

It has been postulated that "intrarenal All" regulates various control functions of the kidney such as regulation of glomerular filtration rate, tubular sodium reabsorption, tubuloglomerular feedback, and vascular resistance. Whether these functions are regulated by blood-borne All generated in renal circulation or by All locally produced in certain renal tissues was not clear.

Mendelsohn's study* with unwashed renal tissues suggested the possibility of the presence of a tissue-bound form of All in the kidney. Immunohistochemical studies demonstrated the coexistence of renin and All-like immunoreactivity in JG cells of rat
FIGURE 1. Serial, adjacent 5 µm paraffin sections of a normal rat kidney stained by the peroxidase-anti-peroxidase method with antibodies to renin (a), angiotensin I (b), and angiotensin II (c). Photographs (a), (b), and (c) contain an identical glomerulus. Immunoprecipitate resulted in dark staining as shown by double arrows. Juxtaglomerular cells in the wall of the afferent arteriole were specifically and strongly stained with antirenin (a) and anti-angiotensin II (c) antisera, but not with anti-angiotensin I antiserum (b). X 200. G = glomerulus; VA = Vas afferens.

This finding raised questions concerning the origin and mechanism of accumulation of AII-like substance in JG cells. The key element in distinguishing these alternative possibilities is the presence of AII in the AII-containing cells.

The substance stained by antibodies to AII must be AII or its breakdown derivatives since it can be detected only by the administration of CEI, and its detectability is improved markedly by protease inhibitors. In either case, the result indicates the presence of AII in JG cells. The colocalization of renin, AII- and AII-like immunoreactivities in JG cells of renal cortex of CEI-treated rats demonstrated in the present study strongly supports an intracellular formation of AII via AII in these cells. The failure to observe AII in the untreated rat may be interpreted as the result of its rapid conversion to AII, presumably by an intracellular mechanism. The fact that strong AII-like immunoreactivity was observed both before and during treatment with CEI, whereas AII-like immunoreactivity was detected only during the treatment and by the use of tissue perfusate-containing protease inhibitors, provides a strong verification of specificity or lack of crossreactivity of anti-AII and anti-AII an-
CEI did not reduce the number of JG apparatus with AII-like immunoreactivity, and that the proportion of the A1-positive cells was smaller than that of the AII-positive cells. It is possible that small peptides such as A1 or AII escaped detection by immunohistochemical technique. Localization of angiotensins by this method has been known to be very difficult. This technique, though highly sensitive, requires a certain threshold level of a tissue antigen for its detection. Low molecular weight antigens like angiotensins are lost in large quantities during the processing of tissues, which involves perfusion, formaldehyde fixation, tibodies in agreement with the findings of competitive binding assays.

The effect of CEI is not instantaneous in spite of large doses of CEI which should effectively block most of extracellular angiotensin-converting enzyme very rapidly. The slow buildup in the intracellular concentration of A1-like immunoreactivity suggests slow accumulation of CEI within JG cells or slow expression of its effect. CEI does not seem to inhibit the intracellular conversion of A1 to AII completely, as indicated by the observations that not all renin-positive JG apparatus were stained by antibodies to A1, that

Figure 2. Serial, adjacent 5 μm paraffin sections of a kidney from a rat chronically fed with angiotensin-converting enzyme inhibitor captopril stained by the peroxidase-antiperoxidase method by the same way as in figure 1. Photographs (a), (b), and (c) contain an identical glomerulus. Immunoprecipitate results in dark staining as indicated by double arrows. Positive staining with anti-angiotensin I antiserum (b) was noted as well as with antirenin (a) and anti-angiotensin II antisera (c) in juxtaglomerular cells in the wall of the afferent arteriole. X 200. G = glomerulus; VA = Vas afferens.
dehydration, and rehydration. Although studies with
the immunohistochemical method do not permit
quantitative determination of antigens, it is not likely
that the concentration of the AI-like immunoreactive
substance was suddenly increased due to tissue disrup-
tion possible caused by the toxicity of a high dose of
CEI concentration. If such toxicity caused tissue disrup-
tion and resulted in the accumulation of AI-like
immunoreactivity, it should have been observed in
other types of cells as well. Experimental results indi-
cate that AI-like immunoreactivity was always ac-
companied by renin immunoreactivity and was con-
fined to the juxtaglomerular region of afferent
arterioles. This specific localization of AI-like
immunoreactivity seems to rule out the possibility of
nonspecific penetration of AI due to its high plasma
concentration induced by CEI.

The concentration of captopril used in the present
study is comparable with those used in pharmacolog-
ical studies of hypertensive rats by other inves-
tigators. In these studies, progressive effects of
captopril on the blood pressure of rats with renin-
independent forms of hypertension were noted. A
similar progressive effect on human essential hyper-
tension was reported. The present observation of
slow and progressive effects of CEI on intracellular
AI-like immunoreactivity suggests the possibility that
the intriguing normalization of blood pressure
observed in renin-independent hypertension may be
related to the inhibition of intracellular renin-angio-
tensin systems in certain tissues, which may include
that of the kidney. These considerations support the
interpretation that AI immunoreactivity confined to
renin-containing JG cells in the rat kidney indicates an
intracellualr pathway for the formation of AI in JG
cells. The concept of an intracellular pathway for the
formation of AI is supported by the observation of
renin, AI, and AI in cloned cultured rat JG cells (un-
published results). Cloned neuroblastoma cells
possessing properties of differentiated neuronal cells
in in vitro culture have been shown to contain renin,
AI, and converting enzyme. The presence of renin and angiotensinogen have been reported in aortic smooth muscle cells. The presence of AI and renin have been reported in the adrenal gland. Thus, accumulating evidence points to a wide-spread intracellular mechanism of angiotensin formation in various endocrine, neuroendocrine, and vascular tissues. These findings suggest that, when released near tissues with AI receptors, the mechanism may be an endocrine or paracrine hormone, which plays a role in elevating blood pressure in the "renin-
independent forms" of hypertension.

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