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 unfleshed 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

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

Immunohistochemical Staining

Serial sections (4-5 μm thick) were mounted on glass slides coated with gelatin chromealum, 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 preincubation 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-AI antisera (fig. 1 c), but no immunostaining was seen with anti-Al antisera (fig. 1 b). 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 AI-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-AI, 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 perfuse (PBS) to prevent peptidolytic destruction of AI. The inhibitors dramatically improved the detection of AI-like immunoreactivity by increasing the number of AI 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, AI and All, respectively, were counted (table 1). Of 400-600 renin-positive JG apparatus, approximately 14% became Al-positive by captopril administration for 2 weeks and 16% with MK-421. Conversely, whenever AI-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 AI locally produced in certain renal tissues was not clear.

Mendelsohn’s study 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 kidneys of rats treated with MK-421 or captopril.
FIGURE 1. Serial, adjacent 5 μm paraffin sections of a normal rat kidney stained by the peroxidase-antiperoxidase 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). × 200. G = glomerulus; VA = Vas afferens.

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

The substance stained by antibodies to AI must be AI 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 AI in JG cells. The colocalization of renin, AI- and All-like immunoreactivities in JG cells of renal cortex of CEI-treated rats demonstrated in the present study strongly supports an intracellular formation of All via AI in these cells. The failure to observe AI in the untreated rat may be interpreted as the result of its rapid conversion to All, presumably by an intracellular mechanism. The fact that strong All-like immunoreactivity was observed both before and during treatment with CEI, whereas AI-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-AI and anti-All an-
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.

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 AI 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,
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 disruption possibly caused by the toxicity of a high dose of CEI concentration. If such toxicity caused tissue disruption and resulted in the accumulation of AI-like immunoreactivity, it should have been observed in other types of cells as well. Experimental results indicate that AI-like immunoreactivity was always accompanied by renin immunoreactivity and was confined 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 pharmacological studies of hypertensive rats by other investigators. 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 hypertension 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-angiotensin 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 intracellular 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 (unpublished results). Cloned neuroblastoma cells possessing properties of differentiated neuronal cells in vitro culture have been shown to contain renin, AI, and angiotensin II, and converting enzyme. The presence of renin and angiotensinogen have been reported in aortic smooth muscle cells. The presence of AII 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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