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HIGH AFFINITY BINDING OF RAMIPRILAT ON ISOLATED HUMAN GLOMERULI

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Abstract—Evidence for angiotensin-converting enzyme (ACE) on isolated human glomeruli was furnished by specific binding of tritium-labeled ramiprilat, a potent inhibitor of ACE.

^{3}H-ramprilat bound to isolated glomeruli, depending on time and temperature displaying a K_D of 3.8 nmol/l and a B_{max} of 853 fmol/mg protein. Specific binding represented more than 90% of total binding. Dissociation occurred rapidly after dilution of the sample with incubation buffer or after addition of an excess of unlabeled inhibitor. Binding of ^{3}H-ramiprilat was also inhibited by increasing concentrations of enalaprilat, another ACE-inhibitor or by preincubation of the glomeruli with polyclonal antibodies against ACE.

ACE is a zinc-containing enzyme. Addition of EGTA to the assay, which chelates zinc ions, completely inhibited binding. This inhibitory effect of EGTA was reversed by divalent Zn^{2+} and Ca^{2+} ions but not by magnesium.

Binding of ^{3}H-ramiprilat to isolated glomeruli was maximal when the pH of the assay medium was brought to pH 8.

In conclusion, the binding of ^{3}H-ramiprilat to isolated human glomeruli is specific and resembles the characteristics which have been found earlier for enzyme activity of ACE. Thus, binding of ^{3}H-ramiprilat to isolated glomeruli can be assumed to be directed to ACE.

With a classical immunofluorescent technique, it is impossible to show the presence of converting enzyme within human normal glomeruli. However, the kidney is a rich source of different kininases, which are present in both the cortex and the outer medulla [1, 2]. Kininase II, being identical with angiotensin-converting enzyme (ACE), was purified from renal cortex [3]. It was further shown that ANG II, which plays an important role in the autoregulation of the kidney [4, 5], is generated in the renal tissue [6].

Moreover, ANG II-receptors have been found on isolated glomeruli [7]. Recently, it has been shown that tritiated perindoprilat, a converting-enzyme inhibitor (CEI), was able to bind specifically to human isolated glomeruli suggesting the presence of ACE in this tissue [8].

The aim of this work was to confirm this result using tritiated ramiprilat, the active metabolite of a highly potent CEI [9]. Furthermore, we planned to study the effect of zinc ions chelation upon the binding of this compound to human isolated glomeruli in order to have a further indirect argument for the presence of ACE, which is a well-known zinc-containing enzyme.

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MATERIALS AND METHODS

Materials. Tritiated ramiprilat (Hoe 498-^{3}H-diacid) was synthesized in the radiochemical laboratories of the Hoechst AG (Frankfurt, F.R.G.) and had a specific activity of 55.4 Ci/mmol. The compound was bilabeled and purified with HPLC.

Preparation of isolated glomeruli. Adult human kidneys were used in this study. They were kindly provided by France Transplant (Hôpital St Louis, Paris) after having been judged unsuitable for transplantation. According to the information we were given, all kidneys were perfused with Collin's solution via the renal artery (500 ml in about 4–5 min), then immersed in ice-cold Collin's medium as for transplantation. The composition of Collin's solution is: K_2HPO_4, 42.5 mM and glucose, 150 mM. The kidneys were stored under these conditions for 36–48 hr until the experiments were started.

Human glomeruli were prepared as follows. Minced renal cortex was mildly pressed through (successively) a 150-μm sieve, which excludes the tubules, and a 75-μm sieve which retained the glomeruli. Glomeruli were suspended in ice-cold Tris–HCl buffer, pH 7.4, containing 125 mM NaCl, 10 mM KCl, 10 mM sodium acetate and 5 mM glucose (buffer A). The suspension was passed through a 25-gauge needle and centrifuged at 120 *g* for 90 sec. The supernate was discarded, the pellet resuspended in the buffer and passed again through the needle

and centrifuged. This operation was repeated 2 times. The final pellet consisted of isolated, decapsulated glomeruli with less than 2% tubular contamination as evaluated under light microscopy. No afferent or efferent arterioles were observed in the preparation.

Binding experiments. Buffer A, 60 μl, containing 41 μg of glomerular protein was added to 40 μl of buffer A containing [^{3}H]-ramiprilat. The final dilution of this tracer was 2–2.3 nmol/l (approx. 400 Bq). Bovine serum albumin was added to prevent adsorption of the tritiated drug to the walls of the incubation tube. Incubation was carried out in a total volume of 100 μl at 22° for 30 min if not otherwise indicated. At the end of the incubation period, 3 ml of chilled buffer A were added and the total volume was passed through a Schleicher & Schüll filter (BA 85) positioned over a vacuum. The filters were washed twice with 3 ml of chilled buffer A, and subsequently added to 10 ml of scintillator (Quicksz-int 2000) followed by shaking for 2 hr. The [^{3}H]-radioactivity was counted in a Beckman LS 1801 liquid scintillation counter with external standard and dpm-calculation. The blank value corresponding to the radioactivity absorbed onto the filter in the absence of glomeruli was approximately 0.2% of total radioactivity. Non-specific binding onto glomeruli was measured in the presence of 20 μM unlabeled ramiprilat and subtracted from total binding to obtain specific binding.

Binding of ^{3}H-ramiprilat has also been measured after a 2-hr preincubation on ice with polyclonal antibodies against ACE at a 1:50 final dilution.

Protein determinations were performed using the method of Lowry *et al.* [11].

RESULTS

The binding of the tritiated ACE inhibitory drug ramiprilat, which is the active metabolite of the prodrug ramipril was measured to increasing amounts of glomerular protein. As is evident from Fig. 1, binding of ^{3}H-ramiprilat was linear over the whole range of glomerular protein concentration (5–1500 μg/ml) when incubated for 30 min. Non-specific binding did not significantly increase and was in the samples with lower protein entirely due to physical binding to the membrane filters as measured by binding in the test system which did not contain binding protein.

The ^{3}H-ramiprilat binding was measured as a function of time. The amount of the drug specifically bound increased with time. It did not reach a plateau after one hour (Fig. 2) but tended to decrease after that time (data not shown). Addition of an excess of unlabeled ramiprilat dissociated the drug-binder complex, since bound radioactivity decreased to about 50% of the initial value 15 min later. This decrease in bound radioactivity represented dissociation of ^{3}H-ramiprilat from its binding site and not degradation of the tracer or binding site, or both, as demonstrated by the persistence of binding in the absence of an excess of unlabelled molecules (Fig. 2). Binding at 4° had a smaller slope in the first minutes and was only about 50% of the binding at 22° after 1 hr (Fig. 2).

Binding of ^{3}H-ramiprilat to isolated glomeruli was also easily reversible upon dilution of the samples after a preincubation of the samples for 30 min at 22°. After addition of an excess of ice-cold incubation medium, the tracer was progressively dissociated from its binder and binding decreased to 50% of its initial value after 10 min (Fig. 3).

Competitive inhibition of binding of ^{3}H-ramiprilat was observed in the presence of increasing concentrations of unlabelled ramiprilat (Fig. 4a).

At a concentration of 1 μmol/l unlabeled ramiprilat, the binding of ^{3}H-ramiprilat was almost completely reversed to filter blank, whereas 50% inhibition was received at 7.2×10^{-9} mol/l. We used the Scatchard transformation of the data (bound-to-free ramiprilat versus the concentration of bound ramiprilat) to calculate the affinity of binding and the number of binding sites. K_D and number of sites were 3.84 nmol/l and 853 fmol/mg protein, respectively.

^{3}H-ramiprilat binding was also inhibited by

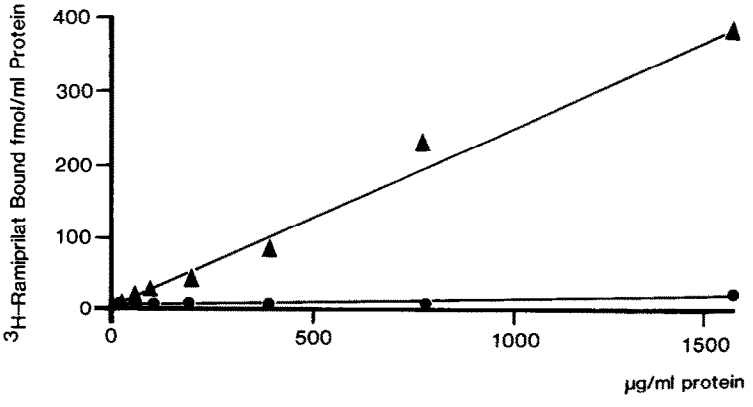

Fig. 1. Binding of ^{3}H-ramiprilat to isolated glomeruli as a function of the concentration of glomerular protein (▲). Lower line (●) represents binding in the presence of excess unlabeled ramiprilat.

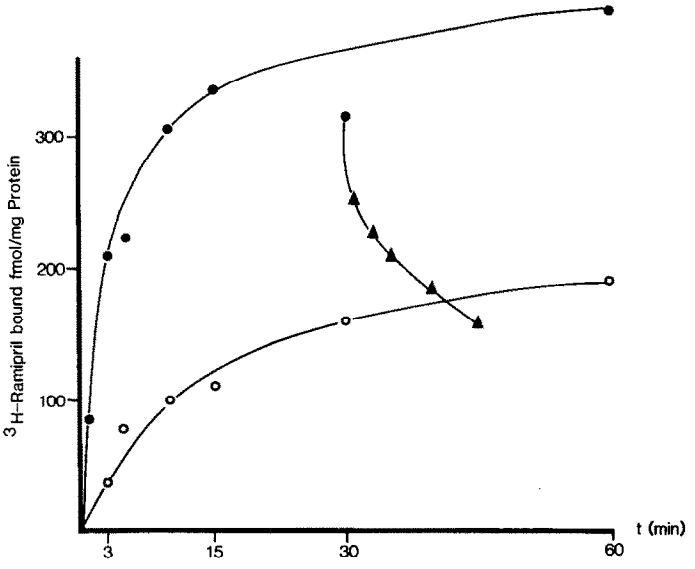

Fig. 2. Time-course of ^{3}H-ramiprilat binding to isolated glomeruli at 22° (●) and at 4° (○). Dissociation of tracer from its receptor (▲) was obtained after addition of 2 μM unlabeled ramiprilat.

increasing concentrations of enalaprilat, another non-sulfhydryl ACE inhibitor (Fig. 4b). The 50% inhibition of radioactivity bound was obtained at a concentration of 1×10^{-8} mol/l for this compound.

When isolated human glomeruli were preincubated with polyclonal antiserum against ACE, binding of ^{3}H-ramiprilat decreased from 20 ± 4.6 to 7.4 ± 1.0 fmol/mg protein, which represents an inhibition of 63%.

ACE is a zinc-containing enzyme. Addition of EGTA in increasing concentration to the incubation medium, which lowers free concentrations of cations by forming complexes, inhibited ^{3}H-ramiprilat binding. This concentration–effect curve was shifted to the right when free Zn^{2+} concentration was elevated by the addition of $ZnCl_2$ to the samples (Fig. 5).

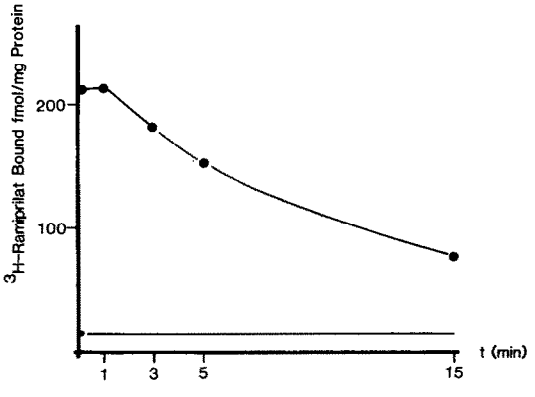

Fig. 3. Dissociation of ^{3}H-ramiprilat from isolated glomeruli after sample-dilution. Isolated glomeruli have been preincubated with ^{3}H-ramiprilat in a volume of 100 μl for 30 min and then been diluted with 3 ml of buffer. Lower line represents binding in the presence of excess unlabeled ramiprilat.

Beside addition of Zn^{2+} the supplement of Ca^{2+} had the same effect (Table 1). In contrast, Mg^{2+} was unable to oppose the inhibitory effect of EGTA.

From several reports it is evident that the enzyme activity of ACE depends on pH [12, 13]. As shown in Fig. 6, this fact corresponds to our finding that binding of ^{3}H-ramiprilat is maximized when the pH of the sample is brought to pH 8.

Addition of zinc chloride did not modify the binding except for a slight elevation of the curve in Fig. 6 (data not shown).

DISCUSSION

The principle of binding ligands to proteins has been widely used to study their anatomical localization. Antibodies to ACE have been used to demonstrate its localization in the pulmonary vascular endothelium [14]. More recently, Mendelsohn [15] used ^{125}I-MK 351A autoradiography to demonstrate the location of ACE in the central nervous system.

Inhibitor binding to membrane-bound ACE was initially described by Strittmatter *et al.* [16] using ^{3}H-captopril. Captopril is not an ideal radioligand as it has a mixed competitive and non-competitive pattern of ACE inhibition [17], and may form dimers via its sulphydryl group binding to endogenous reducing compounds such as cysteine and methionine [18].

Ramiprilat, which is the active compound of the ethylester ramipril, does not contain a thiol element and thus is not associated with problems of instability or high non-specific binding. The advantages of binding a radiolabeled inhibitor instead of measuring catalytic activity include simplicity, specificity and sensitivity, as well as lack of interference from other enzymes.

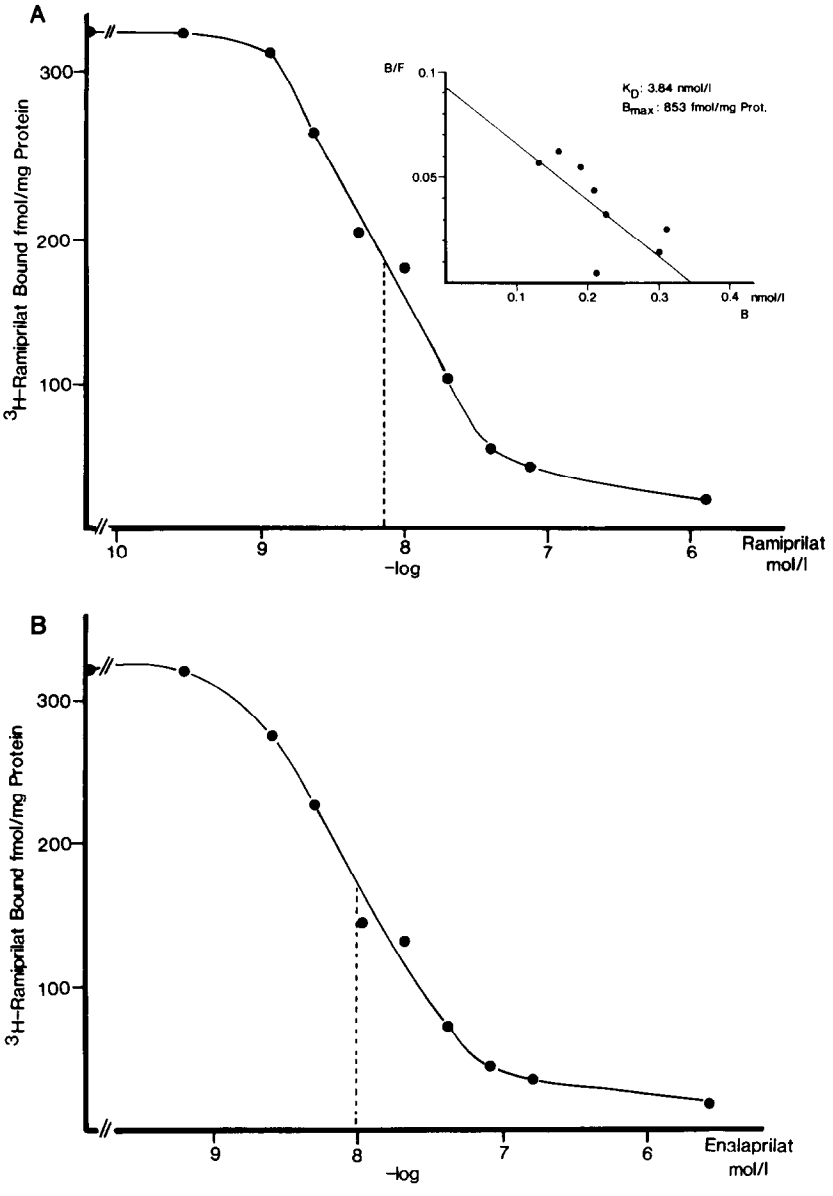

Fig. 4. Competitive inhibition of ^{3}H-ramiprilat binding to isolated glomeruli at increasing concentrations of unlabeled ramiprilat (A) or enalaprilat (B). Dashed line indicates concentration corresponding to 50% inhibition. Scatchard's transformation of data is shown in inset.

The specificity of our assay system was shown by the dose-dependent displacement of bound ^{3}H-ramiprilat from ACE by the other ACE inhibitor enalaprilat. Moreover, binding of the labeled ACE inhibitor was reversible by unlabeled inhibitor independent of the degradation of either hormone or binding site. Finally, preincubation of isolated human glomeruli with polyclonal antibodies directed against ACE inhibited ^{3}H-ramiprilat binding.

Saturation binding was difficult to reach since above one hour of incubation at 22° binding tended to decrease, presumably because of ACE inactivation. These problems were less severe when incubation was performed at 4°, but binding was only 50% as much was bound at 22°, which is in accordance with the finding of Fyhrquist *et al.* [19] who showed a reduction of binding of ^{125}I-MK 351A (a tyrosyl analog of enalapril) to serum ACE of 55% when incubated at 4° instead of 37° independent of how long the incubation lasted. This group also showed that in binding of ^{125}I-MK 351A to serum ACE at 37° an incubation of 4 hr was necessary to reach equilibrium. Due to these difficulties, K_D calculated from Scatchard plot of our data might not reflect true values.

In earlier investigations [12], the catalytic activity of ACE could be completely inhibited by chelating the zinc ions with EDTA. After inactivation of ACE

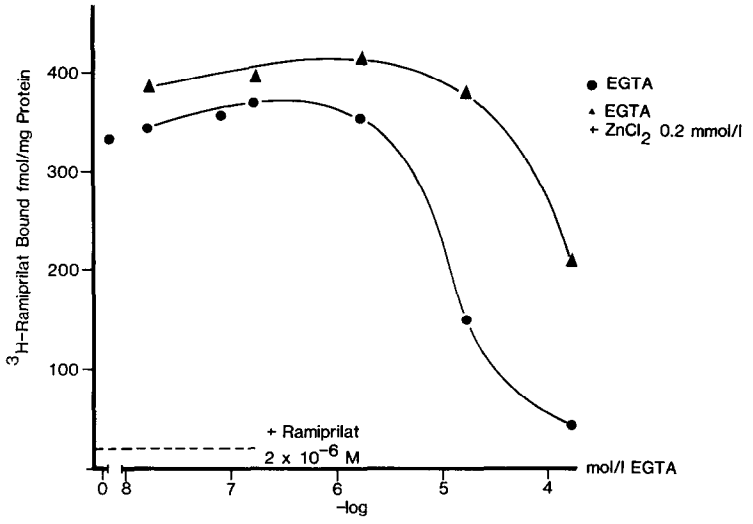

Fig. 5. Inhibition of ^{3}H-ramiprilat binding by increasing amounts of EGTA (●) and reversion of this effect by $ZnCl_2$ (▲). Glomeruli have been incubated with ^{3}H-ramiprilat and EGTA in the absence and in the presence of 0.2 mmol/l $ZnCl_2$ for 30 min at 22°. Dashed line represents binding of ^{3}H-ramiprilat at high concentrations of unlabeled ramiprilat.

by removing all zinc by prolonged dialysis against 1 mM EDTA the enzyme could be reactivated by addition of zinc or manganese to the enzyme. In contrast, addition of calcium or magnesium ions were unable to restore the enzyme activity. In our experiments radioinhibitor binding to isolated glomeruli was also inhibited upon addition of the chelate-forming agent EGTA. EGTA in increasing concentrations decreased labeled ramiprilat binding by reducing the free concentration of Zn^{2+}. This curve was shifted to the right in the presence of zinc chloride. However, care must be taken regarding the concentration given in the figure, since part of the Zn^{2+} seemed to be precipitated by carbonate which is formed by atmospheric CO_2 in aqueous solutions. In contrast to Cushman and Cheung [12] who could not reactivate the zinc-depleted enzyme with Ca^{2+}, in our assay Ca^{2+} was able to overcome the inhibition of ^{3}H-ramiprilat-binding by EGTA. This difference could result from a variation in the experimental procedure. In the experiment of Cushman and Cheung zinc was removed out of the enzyme solution by dialysis against EDTA, whereas in our assay zinc was not removed but transferred into complexes with EGTA. Addition of Ca^{2+} to our assay might have released Zn^{2+} out of the complex, which then in turn restored ^{3}H-ramiprilat binding. Magnesium did not restore ^{3}H-ramiprilat binding after inhibition by EGTA, which is in agreement with the results of Cushman and Cheung regarding the enzyme activity of ACE.

Binding of ^{3}H-ramiprilat in the presence of excess of Zn^{2+} results in a 30% increase in binding. Part of the ACE is probably lacking a Zn^{2+} ion under control conditions due to the preparation and incubation of the glomeruli in a buffer which is devoid of divalent cations.

The pH-optimum of inhibitor-binding, 8.0, differed slightly from the value, 7.0, found by Cushman and Cheung for enzyme activity [12] but was in

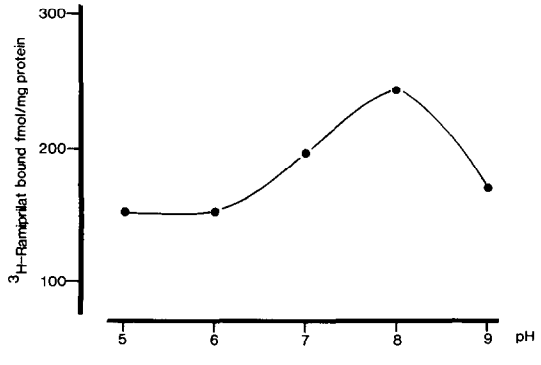

Fig. 6. Binding of ^{3}H-ramiprilat as a function of pH. Binding of ^{3}H-ramiprilat was performed for 30 min at 22° as described in Materials and Methods, with the exception that Tris–HCl has been replaced by Tris–phosphate buffer with varying pH.

Table 1. Effects of divalent cations on restoring specific binding of ^{3}H-ramiprilat to isolated glomeruli after inhibition with 5 × 10^{-4} M EGTA

	fmol/mg protein
Control	336 ± 81
EGTA	84 ± 4
EGTA + $ZnCl_2$	488 ± 9
EGTA + $CaCl_2$	435 ± 51
EGTA + $MgCl_2$	121 ± 6
EGTA:	5 × 10^{-4} mol/l
Cations:	2 × 10^{-3} mol/l

Mean ± SD, N = 3.

accordance with the pH optimum reported by Bünning *et al.* [13] for rabbit lung ACE acting on furanoacryloyl-Phe–Gly–Gly.

These data in concert suggest that ^{3}H-ramiprilat binding sites in this preparation are identical to ACE. It confirms the finding of Chansel *et al.* with ^{3}H-S 9780 [8] and adds a further indirect argument by the demonstration of zinc dependency of the ^{3}H-ramiprilat-binding. However, with this work it is not possible to speculate on the localization of ACE within the glomeruli.

Physiologically, intrarenal ACE might be relevant for the autocontrol of the kidney by intrarenal conversion of ANG I to ANG II. This is emphasized by the fact that CEI are more active as renal vasodilators when infused into the renal artery than when infused intravenously [20]. Renin is released from the juxtaglomerular apparatus of the kidney and angiotensinogen, besides circulating in the plasma, is also synthesized in the renal cortex [21]. Therefore, the demonstration of ACE in the glomeruli is another indication that all components of the renin–angiotensin system are present in the kidney for local generation of ANG II, suggesting that ANG II is indeed a local renal hormone.

The available evidence suggests that angiotensin plays an important role in sodium homeostasis not only via aldosterone release but also through control of the renal circulation. The predominant effect occurs as a vasoconstriction of the efferent arteriole which leads to a decrease of the renal plasma flow and an increase of the glomerular filtration pressure. Owing to these opposing effects, the change in glomerular filtration rate is only moderate [22]. Furthermore, ANG II infusion decreases the surface area available for ultrafiltration, which is postulated to be mediated through a contraction of the mesiangial cells [22].

Finally, this paper gives strong evidence of the presence of ACE in the human glomeruli; however, it is still indirect evidence. The only direct way of demonstrating the presence of ACE would be by the identification of the gene or of the specific RNA of this protein, which is not yet done.

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