Specific nuclear binding of angiotensin II
by rat liver and spleen nuclei

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Summary

1. Nuclei were isolated from rat liver and spleen by a standard technique which preserved the nuclear membrane. Electron microscopy, DNA/protein ratios and 5'-nucleotidase determinations confirmed the purity of the preparation.

2. Both liver and spleen nuclei specifically bound 125I-labelled angiotensin II (ANG II) with high affinity ($K_d \approx 1.0 \text{ nmol/l}$). Saralasin ANG II and ANG II competed with tracer for ligand whereas ANG I did so less effectively and neurotensin not at all.

3. These results suggest the presence of specific nuclear intracellular ANG II receptors and raise important questions regarding the mechanism of action of this peptide.

Key words: angiotensin, nuclear binding.

Introduction

The peptide hormone, angiotensin II (ANG II), is known to bind specifically to target cell membrane receptors and thereby initiate at least some of its physiological action [1-4]. There is evidence that many peptide hormones can gain entry to cells [5-7], and there are data suggesting that tritiated ANG II can localize in the nuclear and mitochondrial regions of myocardial and smooth muscle cells [8-10]. This finding has raised the possibility that some 'late' actions of the hormones, such as cellular hypertrophy or protein synthesis, may be regulated by the binding of the peptide to intracellular sites.

In order to investigate the possibility of intracellular ANG II receptors more directly, we isolated nuclei from the liver and spleens of male Wistar-Kyoto (WKY) rats and studied the ability of 125I-labelled ANG II to bind reversibly and specifically to those nuclei.

Materials and methods

Nuclei were purified from the livers and spleens of male WKY rats by modification of the method of Aldridge [11].

Binding studies were performed in one of two ways. The first method employed 125I-labelled ANG II (New England Nuclear Corp., Boston, MA, U.S.A.; 1000-1500 mCi/mg) at concentrations from 0.1 to 5.0 nmol/l. 125I-labelled ANG II was incubated with 100 µl of isolated nuclei for 0-5 to 120 min in Beckman Microfuge tubes. All determinations were in duplicate or triplicate and for each binding point a comparable set of tubes was incubated in the presence of nuclei, tracer and excess (10^{-6} \text{ mol/l}) ANG II.

In a second series of experiments, 125I-labelled ANG II (0.1-4.3 nmol/l) was incubated in the presence of 5-isoleucine ANG II (Sigma Corporation) at concentrations ranging from 0-5 to 5.0 nmol/l. 125I-labelled ANG II was incubated with 100 µl of isolated nuclei for 0-5 to 120 min in Beckman Microfuge tubes. All determinations were in duplicate or triplicate and for each binding point a comparable set of tubes was incubated in the presence of nuclei, tracer and excess (10^{-6} \text{ mol/l}) ANG II. After incubation at 22\degree C, tubes were spun and aliquots of supernatants were removed. Both the supernatant and the remaining pellet were then counted for radioactivity and bound counts calculated. Specific binding was defined as the difference between the binding in the presence and absence of ANG II at 10^{-6} \text{ mol/l}.

In a second series of experiments, 125I-labelled ANG II (0.1-0.3 nmol/l) was incubated in the presence of 5-isoleucine ANG II (Sigma Corporation) at concentrations ranging from 0-5 to 5.0 nmol/l. Once again a Microfuge binding system was employed as described above, and specific binding was defined as the difference in tracer binding obtained in the presence or absence of ANG II at 10^{-6} \text{ mol/l}.

Electron microscopy was performed on nuclear preparations by standard techniques [12].

Protein was measured by a modification of the method of Lowry [13] and DNA by a modification of the method of Burton [14].
5'-Nucleotidase activity was determined by a modification of the method described by Goldfine et al. [15].

In addition, displacement studies were performed by incubating tracer $^{125}$I-labelled ANG II with various concentrations of [des-Asp]-angiotensin II (ANG II), saralasin, ANG I and neurotensin. For each binding point a series of tubes incubated in the presence of ANG II at $10^{-6}$ mol/l was employed to determine non-specific binding.

Finally, cell membranes were prepared by a modification of the method of Ray [16].

**Results**

Transmission electron microscopy revealed that the nuclear preparations studied consisted of undamaged nuclei free of significant cell membrane or cytoplasmic contamination. Nuclear membranes were not removed by the isolation procedure.

Protein/DNA ratio for spleen nuclei was $2.3 \pm 0.4$ and for liver nuclei $3.2 \pm 0.2$. These ratios were consistent with the ratios expected for pure lymphocytes or hepatocytes respectively. 5'-Nucleotidase activity of the nuclear preparations was less than 2% of that of the intact cells, again indicating that the preparations were free of significant membrane contamination.

Studies employing both spleen and liver nuclei incubated in the presence of $^{125}$I-labelled ANG II revealed high affinity saturable binding to both liver and spleen nuclei. Time course studies revealed that the binding to liver nuclei was rapid, with equilibrium being achieved in about 15 min. Therefore binding studies were performed with an incubation time of 25 min. Scatchard analysis revealed a single class of binding sites with an apparent affinity constant of 1.0 nmol/l in spleen and 1.2 nmol/l in liver nuclei.

In studies employing unlabelled ANG II in concentrations ranging from 0.1 to 5.0 nmol/l for displacement of tracer ANG II, similar results were obtained. An affinity constant for spleen nuclei of 1.0 nmol/l was obtained and for hepatic nuclei 1.1 nmol/l (Fig. 1).

Displacement studies revealed that ANG II, ANG III and saralasin were equally effective in competing with $^{125}$I-labelled ANG II for nuclear binding, whereas ANG I was only about one-hundredth as effective and the unrelated peptide neurotensin could not be shown to compete for binding even at a $10^{-6}$ mol/l concentration.

**Discussion**

The competitive displacement studies which we have performed indicated that the nuclear ANG II receptor recognizes not only ANG II, but also ANG III and saralasin. As such, it has many shared characteristics with other target cell membrane receptors, such as those on the arterial smooth muscle and the adrenal zona glomerulosa cell. We detected some competition for ANG II binding by ANG I but this required concentrations of the decapeptide approximately 100 times greater than that of ANG II for comparable levels of displacement. It also remains unclear whether some of the ANG I-induced displacement resulted from conversion in the assay system into ANG II. The finding that the unrelated peptide, neurotensin, does not displace tracer ANG II from nuclei, further confirms the potential physiological significance of the receptor we have identified.

Our contention that the binding we have observed results from the existence of nuclear binding sites is contingent upon the demonstration that the preparations employed were free of membrane contamination. Electron microscopy revealed the preparations to be pure. DNA/protein ratios were consistent with a pure nuclear preparation free of significant membrane contamination. 5'-Nucleotidase measurements demonstrated that the nuclear preparations contained only a small fraction of the cellular content of this cell membrane bound enzyme. Finally, when in additional experiments hepatic and splenic membranes were isolated specifically and binding studies performed using these membrane preparations, a different pattern of binding was detected. Liver membranes were found to degrade tracer ANG II rapidly at $22^\circ$C in the absence of dithiothreitol. However, nuclear preparations did not degrade tracer under these conditions and definite binding could be detected in the absence of dithiothreitol. The apparent affinity constant for hepatic membranes in our system was about 0.32 nmol/l and this again suggested that the hepatic membrane and nuclear sites were distinct.

![Fig. 1. Scatchard analysis of angiotensin II binding to isolated hepatic nuclei from the rat.](image-url)
For spleen membrane it was quite difficult with our techniques reliably to detect specific high affinity ANG II binding. Although in some experiments spleen membrane preparations could be shown to bind ANG II specifically, we found no evidence for sufficient quantities of high affinity ANG II binding sites on spleen membrane to account by contamination for the observed nuclear binding. Thus we have provided multiple lines of evidence to exclude membrane contamination as a cause for our results. Finally, it is possible that the binding we have observed results from the presence of a non-nuclear intracellular receptor which migrates to the nucleus during our isolation procedure. Although our electron microscopy studies revealed no evidence of such contamination of our nuclear pellets, this possibility cannot be entirely excluded.

The present study suggests the existence of nuclear binding sites for ANG II and once again raises the possibility of an intracellular site of action for this hormone. The fact that we have detected these receptors on the nuclei of cells not usually considered to be ANG II target cells additionally raises interesting questions about the role of ANG II in cellular physiology.

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