ANGIOTENSIN II RECEPTOR CLONING AND SIGNALING: PARTICULARLY ON THE SECOND ISOFORM AT₂. Tadashi Inagami. Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146.

The diversity of ANG II mediated actions encompasses numerous tissues and many different modes of action with different durations, and the existence of multiple receptors has been postulated to explain diverse cellular signalling mechanisms. Invention of subtype-specific inhibitors revealed the presence of three major groups of angiotensin II receptors, AT₁, AT₂ and non AT₁-non AT₂ (AT₃). However, studies with subtype specific inhibitors indicate that practically all of the known responses elicited by angiotensin, either short- or long-term, are mediated by the angiotensin II type 1 (AT₁) receptor subtype. Therapeutic studies using various inhibitors of the renin-angiotensin system have indicated that this system is important in a large subpopulation of patients with essential hypertension and SHR. Again, AT₁-specific receptor antagonists are effective in correcting blood pressure in these hypertensive populations. The molecular basis of the receptor diversity has been very difficult to determine. Attempts to purify solubilized receptors have failed because angiotensin II receptors are very unstable. The problem has been compounded by the possibility that the *mas* oncogene product may be an angiotensin II receptor and by the finding of stable cytosolic angiotensin II binding proteins.

Recent development of the expression cloning technique using mammalian cells has now made it possible to address longstanding issues concerning the structure of these unstable and complex receptors. Thus, AT₁ (type 1 angiotensin II receptor) was expression-cloned from cultured bovine adrenocortical cells and rat vascular smooth muscle cells, which was rapidly followed by hybridization cloning of rat AT₁ cDNA from the kidney of spontaneously hypertensive rats, human hepatic cDNA, human genomic DNA and mouse genomic DNA. In rodents, rat pituitary and adrenal tissue contained a variant of AT₁, which are now designated as AT_{1B} to distinguish it from the vascular, renal and hepatic receptor AT_{1A}. Both AT_{1A} and AT_{1B} were found to have typical structural features of a seven-transmembrane domain receptor. Although these receptors are closely related in base or amino acid sequence (approximately 95% identical in amino acid sequence). However, there seems to be important structural differences in the third cytosolic loop, which plays an important role in interacting with G-proteins.

Type 2 angiotensin II receptor, AT₂, had been considered to be unrelated to AT₁ because of its lack of response to GTP and its stable analog GTPγS. This indicated the possible absence of coupling to a G-protein. Its mRNA did not show cross-reaction with cDNA for AT₁. Again, expression-cloning was called for, but the rapid mutation of AT₂ cDNA in various expression vectors during manipulation presented considerable difficulty to the cloning work. Using conditions to minimize the mutation and stringent size separation of cDNA by K-acetate gradient

centrifugation we were able to isolate a cDNA insert in pCDNAI from the rat pheochromocytoma cell line PC12w.

The amino acid sequence of rat AT₂ deduced from the cloned cDNA was found unexpectedly to have the structural motifs of a seven membrane spanning domain receptor with 363 amino acid residues which corresponded to a theoretical molecular weight of 41,303. It also contained five potential N-glycosylation sites in the amino terminal extracellular domain. The difference of 30,000 from the molecular weight (71,000) of the native receptor isolated from PC12w cell plasma membrane by affinity chromatography may be accounted for by carbohydrate chains on these glycosylation sites. In AT₁ there are 3 potential N-glycosylation sites, one in the N-terminal extracellular domain and two in the second extracellular loop. Three carbohydrate chains may account for the difference between the native molecular weight of 41,000. The comparison of the amino acid sequence of rat AT_{1A} and AT₂ revealed only 32% sequence identity, and if anything, they are only remotely related.

Although both of the isoforms turned out to be seven transmembrane domain receptors, a marked difference in their signalling and physiological functions are obvious. Their localization and regulation of expression share little commonality.

The typical and most prominent action of angiotensin II is the Gq mediated activation of PLC, which is usually followed by an opening of an L-type Ca²⁺ channel which can be blocked by its specific blockers such as dihydropyridines. It was also shown to inhibit adenylyl cyclase *via* a Gi mediated mechanism. Human AT₁ receptor and rat AT_{1A} receptor stably expressed in CHO cells were shown to mediate all of the three responses. Since CHO cells lack endogenous angiotensin II receptor, and since only one type of angiotensin II receptor was expressed, it is clear that the AT₁ receptor can interact with three types of G proteins. Between the two AT₁ subtypes, AT_{1A} and AT_{1B} in rodents amino acid sequences of the third cytosolic loop domain show a few important diversity. This domain plays a major role in activating the G proteins. The amino acid sequences of human or bovine AT₁ more closely resembles that of rat AT_{1A} than AT_{1B}. Thus, rat AT_{1B} may have a unique feature, somewhat different from that of AT_{1A} or probably from human AT₁.

The function of AT₂ has been controversial. Using cells abundantly expressing this receptor, it has been shown not to increase [Ca²⁺]_i, nor change cAMP. However, disagreements exist as to whether angiotensin II inhibits cGMP production, and as to whether it stimulates or suppresses phosphotyrosine phosphatase. A biphasic concentration dependency on Ang II seems to explain the stimulation or inhibition of the phosphatase. The cloned and stably expressed AT₂ in COS-7 cells did not show a reduction in cGMP in response to angiotensin II, nor in PC12w cells which express only AT₂ but not AT₁.

To gain insight into the overall role of AT₂, we deleted the AT₂ gene in the mouse by targeted homologous recombination technique. The AT₂ gene is present

on the X chromosome and AT_2 deleted male mice were produced readily as germ line transmission took place.

Interestingly, AT_2 -gene deleted mice showed an appreciable elevation of basal blood pressure by 25 mmHg compared with AT_2 gene intact litter mates. Furthermore, infusion of Ang II at subpressor dose into enalapril-treated mice without endogenous Ang II, uncovered marked sensitization to Ang II in AT_2 gene-deleted mice compared to AT_2 intact mice. Angiotensinogen and AT_{1A} had been deleted. They show lowering of blood pressure. Since AT_2 is less dominant compared to AT_1 , the present results indicate that AT_2 functions to blunt the pressor effects of Ang II through AT_1 . AT_2 also has an interesting effect in renal sodium reabsorption, and the AT_2 deleted mice seem to have a partial impairment of Na-retention. Thus, various techniques including AT_2 gene knockout experiments are revealing several interesting aspects of Ang II actions hitherto unknown, and will make an important contribution to our understanding of control of blood pressure and organ damage by Ang II.