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Angiotensin II stimulates angiotensinogen synthesis in hepatocytes by a pertussis toxin-sensitive mechanism

C. Klett, F. Muller, P. Gierschik and E. Hackenthal

Department of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 6900 Heidelberg, FRG

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The role of intracellular messengers in the stimulatory effect of angiotensin II on angiotensinogen synthesis and secretion in hepatocytes was examined. Angiotensinogen secretion was not influenced by modulators of intracellular calcium (calmidazolium, A 23187, Bay K 8644, methoxamine). In contrast, agents decreasing intracellular cAMP (angiotensin II, guanfacine) stimulated, and those increasing cAMP (isoproterenol, glucagon, forskolin) depressed angiotensinogen secretion. An inverse relationship was also observed between cAMP and angiotensinogen mRNA. Pretreatment of hepatocytes with pertussis toxin abolished the stimulation by angiotensin II. It is concluded that angiotensin II-induced stimulation of angiotensinogen synthesis is initiated by inhibition of adenylate cyclase.

Angiotensinogen synthesis; Angiotensin II; Pertussis toxin; Adenosine monophosphate, 3', 5'-cyclic; (Rat hepatocyte)

1. INTRODUCTION

The glycoprotein angiotensinogen serves as the substrate of renin (EC 3.4.23.15) for the generation of angiotensin I, which is subsequently converted to angiotensin II. The rate of angiotensinogen secretion by the liver has a direct influence on steady-state concentrations of angiotensin II in the circulation [1]. Conversely, angiotensin II seems to have a stimulatory effect on angiotensinogen secretion, at least under pathophysiological conditions [2,3]. This response to exogenous angiotensin II, which may reflect the effector limb of a positive feedback loop, has also been observed in the isolated liver [4,5], and isolated hepatocytes [5,6].

We have previously shown in isolated hepatocytes that angiotensin II-induced secretion of angiotensinogen is preceded by an increase in angiotensinogen mRNA [7]. In the present study, we examined which of the known signal transduction pathways for angiotensin II in hepatocytes mediates this response.

2. MATERIALS AND METHODS

2.1. *Preparation of hepatocytes and incubation conditions*

Hepatocytes were isolated from livers of male Sprague-Dawley rats weighing 200–280 g by the collagenase technique of Seglen [8] with several modifications described previously [5,9]. Cells were suspended in minimal essential medium (MEM) containing Earle's salts to a density of 1.5×10^6 cells/ml, and incubated in 30 ml aliquots in 400 ml round-bottom flasks under an atmosphere of O_2/CO_2 (95:5) at 37°C and 60/min oscillations. At the beginning of incubation, 0.125 μCi of [U-^{14}C]leucine was added per flask for the estimation of protein synthesis and secretion. At various time intervals, aliquots were taken for the estimation of albumin, angiotensinogen, and leucine-labelled proteins in the cell-free supernatant and of angiotensinogen mRNA, cAMP, and leucine-labelled proteins in the cell pellet.

Experimental additions to hepatocyte incubations were made in a single volume of less than 0.5 ml, except for angiotensin II, which was added by continuous infusion, since this peptide undergoes rapid degradation in hepatocyte suspensions [5,9]. Actual concentrations of angiotensin II in the incubation medium were estimated by radioimmunoassay [5].

2.2. *Pertussis toxin treatment*

Pertussis toxin was purified from the culture broth of Bordetella pertussis (kindly donated by Dr F. Blackkolb, Behringwerke, Marburg, FRG) as described by Sekura [10], except that ammonium sulfate precipitation of the purified toxin was omitted. Hepatocyte suspensions were exposed to pertussis toxin (0.5 μg/ml) for 3 h, and subsequently for 1 h to 90 nM angiotensin II (or vehicle). Immediately before and after 1 h of angiotensin II exposure, samples were taken for the estimation of angiotensinogen mRNA. Incubations without pertussis toxin and/or without angiotensin II served as controls. Treatment of intact cells with pertussis toxin led to an almost complete ($\geq$95%) ADP-ribosylation of the approximately 40 kDa toxin substrate(s) present in hepatocyte membranes, as assessed by [^{32}P]ADP-ribosylation of membrane proteins using pertussis toxin and [^{32}P]NAD.

2.3. *Analytical methods*

Angiotensinogen concentrations were determined by incubating aliquots with an excess of hog renin and estimation of angiotensin I by radioimmunoassay [5].

Angiotensinogen mRNA was measured by liquid hybridization with labelled angiotensinogen cRNA as described by Hellmann et al. [11]. The plasmid used for cell-free transcription of ^{32}P-labelled antisense and unlabelled sense angiotensinogen mRNA was pRAN 2, containing the vector pspt 18, an angiotensinogen 712 bp *Bam*HI cDNA fragment, and the promoters for SP 6 and T 7 polymerases. For details see [8,11].

Albumin was measured with a radioimmunoassay [9].

Incorporation of [^{14}C]leucine into secreted and cellular protein was measured as described [9].

Correspondence address: E. Hackenthal, Department of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, 6900 Heidelberg, FRG

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For the estimation of cAMP, 1 ml aliquots of hepatocyte suspension were lysed by the addition of 2 ml of ethanol. Following centrifugation at 200 × *g* for 3 min, the pellet was reextracted with 1 ml of 60% ethanol, the supernatants were combined, centrifuged at 2000 × *g* for 15 min at 4°C, and evaporated at 60°C in a stream of nitrogen, the residue dissolved in 50 mM phosphate buffer, pH 6.8, and cAMP measured by radioimmunoassay.

2.4. *Materials*

The plasmid used for the cell-free transcription of the hybridization probe was a gift from Drs Ganten and Hellmann, Dept. of Pharmacology, Heidelberg. ^{32}P-labelled nucleotides were from DuPont de Nemour (Bad Homburg, FRG). A 23187 and glucagon were from Sigma (Munich), other peptides from Bachem (Switzerland). Calmidazolium was obtained from Janssen Pharmaceuticals (Neuss), [^{14}C]leucine was from NEN (Dreieich), and [^{125}I]iodide, as well as the cAMP radioimmunoassay kit from Amersham Buchler (Braunschweig, FRG). Bay K 8644 was a gift from Dr R. Gross (Bayer AG, Leverkusen), TPA from Dr Hecker (Deutsches Krebsforschungszentrum, Heidelberg). All other reagents were of analytical grade or the highest purity available.

2.5. *Evaluation of data*

Data are expressed as mean values ± S.E.M. Experimental groups were compared by ANOVA and, if applicable, by Bonferoni's method.

3. RESULTS

3.1. *Modulation of second messengers and angiotensinogen secretion*

The major signal transduction pathway by which angiotensin II influences intracellular functions in hepatocytes seems to be the activation of phospholipase C, release of both inositol trisphosphate and diacylglycerol, and the subsequent increase in intracellular calcium and activation of the Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C), respectively [12,13]. In addition, angiotensin II has been shown to inhibit adenylate cyclase and reduce intracellular cAMP [14,15]. To identify the pathway that mediates the stimulation of angiotensinogen synthesis and secretion, hepatocytes were exposed to agents that mimic or modulate the availability of intracellular messengers. As angiotensinogen secretion starts to increase 2–3 h after exposure to angiotensin II, angiotensinogen secretion was measued during the 4th hour of incubation. The results are summarized in table 1. Angiotensin II (90 nM) induced an almost 2-fold increase in angiotensinogen secretion. Various modifiers of intracellular calcium concentration or availability such as the α_1-adrenoceptor agonist, methoxamine, the calcium ionophore, A 23187, the calcium channel agonist, Bay K 8644, and the calmodulin antagonist, calmidazolium, failed to influence angiotensinogen secretion. Oleylacetylglycerol, which mimicks endogenous diacylglycerol, had no effect on angiotensinogen secretion in concentrations up to 10 μM, when given alone or in combination with A 23187, whereas the protein kinase C activator, TPA (12-*O*-tetradecanoylphorbol-13-acetate), reduced angiotensinogen secretion by 33%.

Table 1

Modulation of intracellular messengers and angiotensinogen

Agent		Angiotensinogen secretion (ng AI/mg wet wt./h)		
		Control	Experimental	% change
Angiotensin II	(90 nM)	106 ± 7	209 ± 9	+97*
A 23187	(5 μM)	142 ± 15	135 ± 15	− 9
Bay K 8644	(5 μM)	238 ± 14	253 ± 7	+ 6
calmidazolium	(5 μM)	137 ± 10	126 ± 7	− 8
methoxamine	(10 μM)	98 ± 3	96 ± 9	− 2
OAG	(10 μM)	121 ± 9	136 ± 8	+12
OAG and A 23187	(10/5 μM)	107 ± 10	114 ± 7	+ 6
TPA	(0.2 μM)	185 ± 6	124 ± 12	−33*
forskolin	(1 μM)	185 ± 6	119 ± 8	−36*
glucagon	(50 nM)	192 ± 10	91 ± 3	−53**
isoprot + IBMX	(5 μM)	112 ± 3	71 ± 4	−37*
guanfacine	(10 μM)	107 ± 5	170 ± 11	+59**

Agents have been added at time zero, and angiotensinogen secretion was measured during the 4th hour in these groups (experimental) in comparison to the respective control incubations (control). OAG = oleylacetylglycerol; IBMX = isobutylmethylxanthine; isoprot = isoproterenol; most agents have been examined at 2–3 concentrations. Only the highest conc. are listed. Mean values ± SEM of 5–6 separate experiments

*$P \leq 0.05$; **$P \leq 0.01$

In contrast to calcium-modulating agents, all substances affecting intracellular cAMP concentrations modified angiotensinogen secretion. Thus, isoproterenol (in combination with the phosphodiesterase inhibitor, isobutylmethylxanthine), glucagon and forskolin reduced angiotensinogen secretion by 37%, 53% and 36%, respectively, whereas the α_2-adrenoceptor agonist, guanfacine, increased angiotensinogen secretion by 59%.

All experimental additions described had no influence on [^{14}C]leucine incorporation into cellular or secreted protein, nor did they affect albumin secretion during the observation period (data not shown).

3.2. *Effect of glucagon and angiotensin II on angiotensinogen mRNA*

The opposing effects of agents stimulating or inhibiting cAMP formation on angiotensinogen secretion are also apparent at the mRNA level. As shown in fig.1, glucagon (5 and 50 nM) reduces angiotensinogen mRNA from 5.7 ± 0.21 to 4.8 ± 0.23, and 3.7 ± 0.14 pg mRNA/μg total RNA, respectively, during 2 h of exposure, whereas angiotensin II (9 and 90 nM) increased angiotensinogen mRNA to 7.8 ± 0.3 and 10.1 ± 0.5 pg/μg RNA, respectively.

3.3. *Relationship between angiotensinogen synthesis and intracellular cAMP*

Direct evidence for an inverse relationship between intracellular cAMP and angiotensinogen synthesis and secretion is given in fig.1. Within 5 min after the addition of angiotensin II (9 and 90 nM) cAMP was (tran-

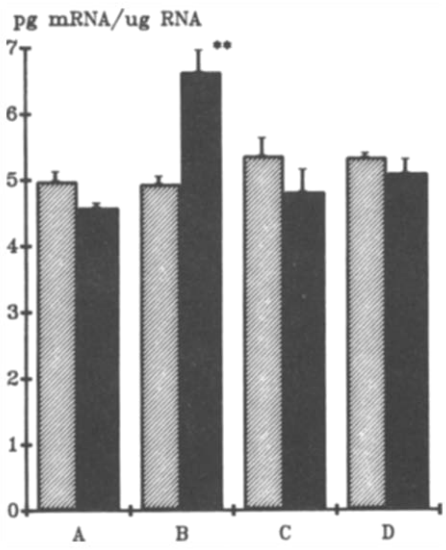

Fig.1. Effects of glucagon and angiotensin II on cAMP, angiotensinogen mRNA and secretion. Glucagon (Glu) or angiotensin II (AII) were added at time zero to hepatocytes as described in section 2. Mean values ± SEM of experiments (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

siently) reduced by 24% and 42%, respectively, whereas glucagon (5 and 50 nM) produced the expected increase in cAMP, which, at 5 min, was about 3- and 6-fold, respectively.

This relationship was further studied in pertussis toxin-treated hepatocytes (fig.2). In control hepatocytes, angiotensinogen mRNA concentration was 5.27 ± 0.15 pg/μg total RNA at time zero, and fell slightly to 5.0 ± 0.14 and 4.6 ± 0.06 pg/μg RNA at 3 and 4 h of incubation, respectively. Addition of 90 nM angiotensin II at 3 h led to an increase from 4.9 ± 0.1 to 6.6 ± 0.43 pg mRNA ($P < 0.01$) within 60 min, whereas in hepatocytes pretreated for 3 h with 0.5 μg/ml of pertussis toxin, the increase in angiotensinogen mRNA by angiotensin II was completely blocked.

4. DISCUSSION

The activation of phospholipase C with the subsequent increase in inositol triphosphate and cytosolic calcium is generally considered the major transmembrane signalling system for angiotensin II in hepatocytes [12,13]. Surprisingly, all pharmacological manipulations of intracellular calcium failed to influence angiotensinogen secretion. Although intracellular calcium was not measured in these experiments, it is unlikely that no changes in intracellular calcium homeostasis occurred with any of the agents used.

Another consequence of the activation of phospholipase C by angiotensin II is the liberation of diacylglycerol and activation of protein kinase C. The failure of oleylacetylglycerol to affect angiotensinogen secretion may be due to insufficient activation of protein kinase C, since the phorbol ester, TPA, a potent activator of protein kinase C, significantly reduced the rate of angiotensinogen secretion. However, this inhibition runs counter to the observed stimulation of angiotensinogen secretion by angiotensin II, if angiotensin II were assumed to control angiotensinogen synthesis through this pathway (see, however, below).

The other transmembrane signalling pathway for angiotensin II in hepatocytes is the coupling of angiotensin II receptors to adenylate cyclase through an inhibitory G_i-protein [14,15]. This type of coupling has also been demonstrated in hepatocytes for α_2-adenoceptor agonists like guanfacine [14]. The data

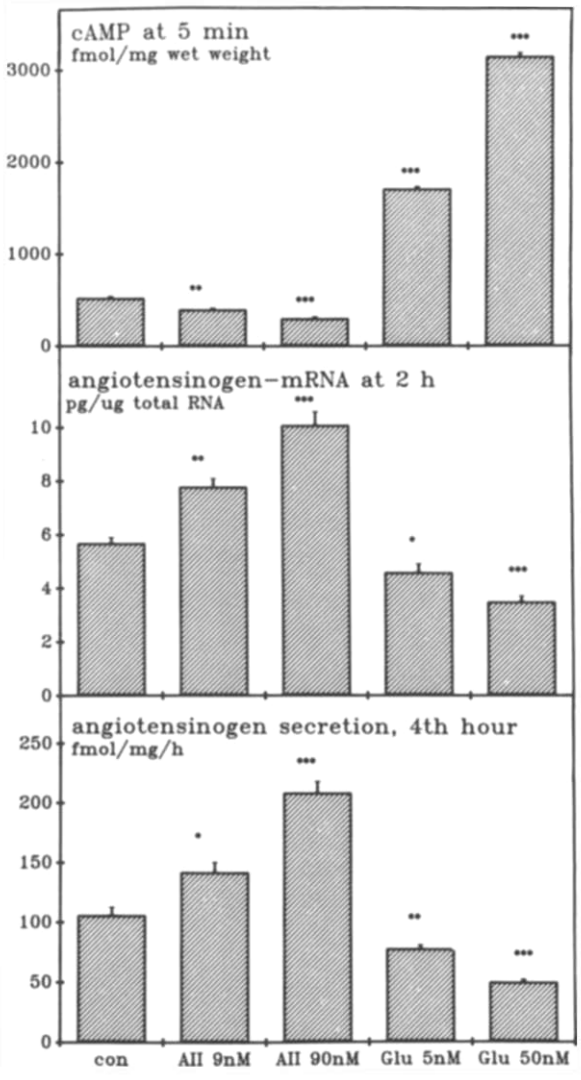

Fig.2. Pertussis toxin prevents the response of angiotensinogen mRNA to angiotensin II. Hepatocytes in groups C and D were pretreated for 3 h with 0.5 μg/ml pertussis toxin (PT). Angiotensin II (AII) 90 nM was added at 3 h to groups B and D. Control hepatocytes received no addition (group A). Angiotensinogen mRNA was measured at 3 (left, light-shaded columns) and 4 h (right, dark columns) and expressed as pg mRNA/μg of total RNA. There were no significant differences in total RNA (**$P \leq 0.01$ for the difference to values before AII).

of table 1 and fig.1 suggest that manipulations of the intracellular concentration of cAMP induce an inverse change in angiotensinogen synthesis and secretion. Since angiotensin II induces both a decrease in cAMP and an increase in angiotensinogen mRNA, it appears possible that inhibition of adenylate cyclase by angiotensin II rather than its calcium-mobilizing effect is the initiating action by which angiotensin II stimulates angiotensinogen synthesis. This view is strongly supported by the observation that pertussis toxin completely abolishes this effect of angiotensin II, since it has been demonstrated in several laboratories that, in hepatocytes, the G_i-protein of the adenylate cyclase complex, but not the G-protein coupling angiotensin II receptors to phospholipase C is pertussis toxin-sensitive (e.g. [15–17]). This interpretation would also help to explain why activation of protein kinase C by TPA inhibits angiotensinogen secretion, as several laboratories have reported that activation of protein kinase C by TPA induces phosphorylation and inactivation of the G_i-protein [18–20]. This would tend to increase adenylate cyclase activity and, consequently, decrease angiotensinogen synthesis.

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