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Acceleration of protein synthesis by angiotensin—Correlation with angiotensin's effect on catecholamine biosynthesis

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ANGIOTENSIN-II has a potent effect in accelerating noradrenaline (NA) biosynthesis in a number of sympathetically innervated tissues.[1–3] This action may have some importance in relation to the homeostatic control mechanisms *in vivo* within the sympathetic neuron, for maintenance of constant levels of endogenous NA, and may possibly be involved in the relationship between the renin–angiotensin system and the development of certain hypertensive states. In a further investigation of the possible mechanism of action of angiotensin in accelerating the biosynthesis of NA, we have found that this peptide has marked effects on protein synthesis. The following results may help to explain not only why angiotensin increases the synthesis of NA, but may also have wider implications in respect to the possible physiological and pathological roles of the renin–angiotensin system.

Guinea pig atria were dissected out from male animals killed by cervical dislocation. The tissues were incubated at 37° for 1 hr in Krebs–Henseleit solution containing either tyrosine-^{14}C (10 mCi/m-mole, 5×10^{-5} M) or tyrosine-^{14}C and leucine-^{3}H (250 mCi/m-mole, 2×10^{-5} M). Where indicated, angiotensin-5-valine amide II (Ciba) was added immediately prior to beginning the incubation. At the end of the incubation period, the atria were blotted and frozen on solid carbon dioxide. The atria were weighed and then homogenized in 15% trichloroacetic acid containing 0·1% disodium edetate. The homogenate was centrifuged for 15 min at 20,000 *g* and the supernatant taken for the isolation of catechols by the alumina column procedure.[4] The tissue and supernatant were maintained at 4° throughout all these procedures.

The residual tissue pellet was extracted with 2 ml of cold 0·4 N perchloric acid and allowed to stand for 1 hr at 4°; the sample was then centrifuged at 20,000 *g* for 10 min and the supernatant discarded. The washed pellet was then re-extracted with 2 ml of 0·4 N perchloric acid at 95° for 20 min to hydrolyze and extract nucleic acids. The sample was cooled and recentrifuged; the hot perchloric acid extract plus a 1-ml wash (0·4 N perchloric acid) of the pellet was kept for pentose analyses by the diphenylamine spectrophotometric method using deoxyadenosine as the standard.[5] Amino acid incorporation into protein was determined by dissolving the final protein pellet in 3 ml of Soluene (Packard). The sample was then counted in 20 ml of a dioxane–toluene–ethanol scintillation fluid[6] in a Packard scintillation counter. Standard procedures for quench and overspill correction were used in experiments with both ^{14}C- and ^{3}H-labeled amino acids. Amino acid incorporation into protein was expressed in terms of dis./min/g wet wt of tissue or in dis./min/nmole of DNA deoxyribose.

Since the actions of many hormones have been shown to be associated with effects on protein synthesis, the possibility that the previous observed actions of angiotensin on NA biosynthesis might be explained by an effect on protein synthesis seemed plausible. When atria were incubated with 5×10^{-7} M angiotensin as previously reported,[1–3] the biosynthesis of NA was 40–100 per cent greater than that of the control tissues. At the same time, it was found that tyrosine incorporation into protein was also elevated to about the same extent by angiotensin (Fig. 1). This observation was suggestive that angiotensin at a concentration (5×10^{-7} M) that was producing a maximal increase in NA synthesis was also increasing protein synthesis. Previous experiments have indicated that angiotensin at concentrations of 10^{-9} to 5×10^{-7} M has no significant effect on the uptake of tyrosine into the guinea pig atria[1,3]. Angiotensin at a higher concentration (10^{-5} M), which produced

no stimulatory effect on NA biosynthesis, did not increase tyrosine incorporation into atrial protein and, in fact, appeared to lead to a significant inhibition of tyrosine incorporation. However, the actual concentration of angiotensin responsible for the observed effect is unknown, since the concentration of the active peptide in the medium is rapidly changing during the incubation period. Experiments with guinea pig atria carried out under conditions similar to those of the above described experiments have demonstrated that 400–600 mg of tissue incubated in 5 ml of Krebs containing

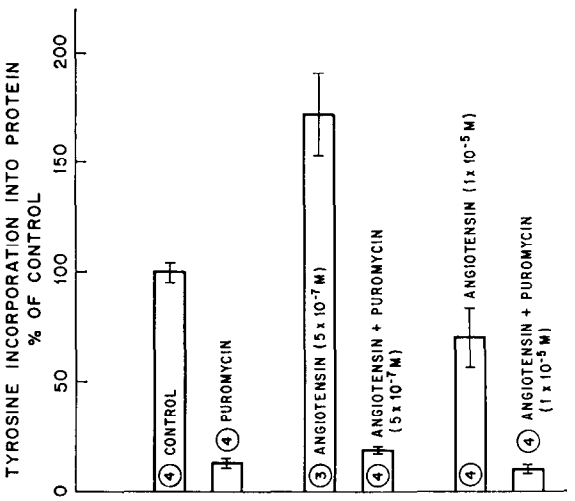

FIG. 1. Effect of angiotensin and puromycin on the incorporation of tyrosine into protein. The vertical bars depict the standard error of the mean. The number of individual experiments is indicated at the bottom of each bar. The incubation with ^{14}C-tyrosine (10 mc/m-mole, 5×10^{-5} M) was for 1 hr. Control incorporation of tyrosine into protein was 323 $\pm$ 13 dis./min/mg of tissue.

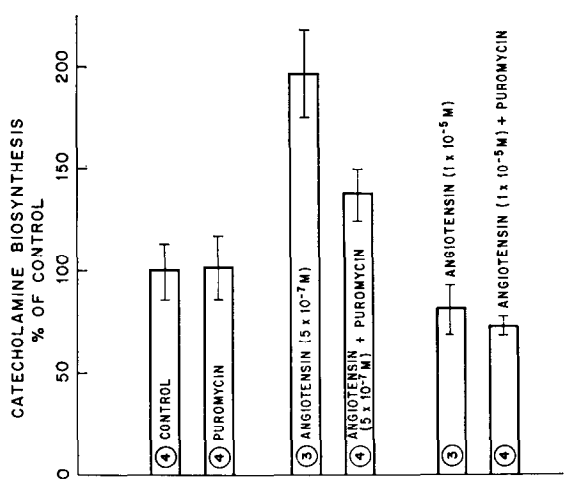

FIG. 2. Effect of puromycin on the angiotensin-induced increase in catecholamine biosynthesis. The vertical bars depict the standard error of the mean. The number of individual experiments is indicated at the bottom of each bar. Control catecholamine synthesis was 19,230 $\pm$ 2735 dis./min/g/hr. Where indicated, puromycin was added immediately prior to incubation at a concentration of 1×10^{-4} M. The atria were incubated with ^{14}C-tyrosine (10 mc/m-mole, 5×10^{-5} M) for 1 hr at 37°.

TABLE 1. EFFECT OF ANGIOTENSIN ON LEUCINE AND TYROSINE INCORPORATION INTO PROTEIN AND ON NORADRENALINE BIOSYNTHESIS IN GUINEA PIG ATRIA

Treatment*	N	Leucine incorp. (dis./min/nmole of DNA deoxyribose)	% Change†	N	Tyrosine incorp. (dis./min/nmole of DNA deoxyribose)	% Change†	NA synthesis (dis./min/g/wet wt)	% Change
None	4	6523 ± 265		8	158 ± 12		20,276 ± 1849	
Puromycin (1×10^{-4} M)	4	594 ± 25‡	−91	4	14 ± 0·3‡	−91	23,821 ± 3935‖	+17
Angiotensin (5×10^{-7} M)	3	9763 ± 1351§	+43	7	235 ± 27§	+49	34,472 ± 5093§	+70
Puromycin (1×10^{-4} M) + Angiotensin (5×10^{-7} M)	4	655 ± 62‡	−90	3	18 ± 1·6‡	−89	21,350 ± 2574‖	+5

* Atria were preincubated at 37° with or without puromycin prior to addition of labeled amino acids and angiotensin where indicated.
† Percentage change when compared to non-treated control atria.
‡ Comparison of drug-treated with control atria, $P < 0{\cdot}001$.
§ Comparison of drug-treated with control atria, $P < 0{\cdot}05$.
‖ Not significant, $P > 0{\cdot}1$.

250 ng angiotensin (5×10^{-8} M) for 30 min resulted in a total loss of biological activity in the rat colon assay.[2]

Since an increase in protein synthesis appeared to be correlated with the observed increase in NA biosynthesis induced by angiotensin, it was of interest to see if blocking protein synthesis would antagonize the angiotensin-induced increase in catecholamine biosynthesis. Puromycin, a potent inhibitor of protein synthesis, in a concentration (1×10^{-4} M) which inhibits tyrosine incorporation into protein by about 75 per cent, and which has no significant effect on the basal rate of NA biosynthesis was found to antagonize significantly the angiotensin-induced increase in NA synthesis (Fig. 2). In fact, additional experiments demonstrated that, if atria are preincubated for 15 min with puromycin prior to addition of angiotensin in order to block protein synthesis more effectively, the angiotensin effect on NA synthesis can be completely abolished (Table 1). In some experiments, another inhibitor of protein synthesis, cycloheximide, was tested for its ability to antagonize the angiotensin-induced increase in NA biosynthesis. Incubation of atria with cycloheximide in a concentration which blocked tyrosine incorporation into protein by more than 90 per cent completely blocked the ability of angiotensin to accelerate NA biosynthesis (Table 2). Cycloheximide alone had no significant effect on NA biosynthesis.

TABLE 2. EFFECT OF CYCLOHEXIMIDE ON THE ACCELERATION OF NORADRENALINE BIOSYNTHESIS PRODUCED BY ANGIOTENSIN

Treatment	N	NA synthesis (dis./min/g wet wt)	% Change*
None	11	22,964 ± 2567	
Cycloheximide (1×10^{-4} M)	4	22,737 ± 2893	−1
Angiotensin (5×10^{-7} M)	16	33,501 ± 3391	+47†
Cycloheximide (1×10^{-4} M) + Angiotensin (5×10^{-7} M)	8	21,384 ± 3306	−6‡

* Percentage change when compared to untreated control atria.
† Significantly different from untreated control, $P < 0{\cdot}05$.
‡ Not significantly different from untreated or cycloheximide-treated atria.

Subsequent experiments demonstrated that the ability of angiotensin to stimulate incorporation of amino acids into protein was not limited to the aromatic amino acid tyrosine. Angiotensin also had a similar stimulatory effect on the incorporation of leucine into atrial protein (Table 1). In this case, the atria were also preincubated with puromycin for 15 min prior to the addition of angiotensin and the labeled amino acids in an attempt to effect a more complete block of protein synthesis. Again the acceleration of NA synthesis (Table 1) by angiotensin was accompanied by a significant increase in the incorporation of both tyrosine and leucine into protein. The effects of angiotensin on catecholamine synthesis were completely abolished in puromycin-treated atria and the basal incorporation of amino acids into protein was reduced by about 90 per cent. Although currently we are unable to state with any certainty the precise site of action of angiotensin on protein synthesis, the present results do show a very close parallel between enhanced protein and NA synthesis. Thus those atria exhibiting the greatest increase in NA synthesis also showed the greatest increase in protein synthesis. However, the magnitude of the effect of angiotensin on protein synthesis as well as the rapid time course of action argues somewhat against a selective action of angiotensin on the sympathetic neurons which make up only a small portion of the total atrial mass. It is, of course, possible that the bulk of amino acid incorporation into protein is occurring outside the sympathetic neuron and that the correlation between the angiotensin-induced increase in NA and protein synthesis is just fortuitous and not directly interrelated. Studies in progress on the effect of angiotensin on chemically sympathectomized atria might help in clarifying this issue.

The dose–response relationship between angiotensin and NA synthesis is very steep.[3] In the guinea pig atria, the maximum acceleration is seen at an angiotensin concentration of approximately 5×10^{-7} M. At concentrations of angiotensin greater than 10^{-6} M this stimulatory effect on the synthesis of NA is lost and at higher concentrations inhibition is often observed. In the present experiments this inhibitory effect on NA synthesis was apparent at 10^{-5} M angiotensin (Fig. 1). Moreover, this concentration also significantly inhibited protein synthesis (Fig. 2). Again, it appears that there is a close correlation between the effects of angiotensin on protein and NA synthesis.

The acceleration of NA synthesis by angiotensin also appears to depend on the presence of the adrenergic terminal plexus. Experiments with sections of the bovine splenic nerve trunk revealed that in this tissue even low concentrations of angiotensin do not increase NA synthesis (Table 3). Higher concentrations led to significant inhibition of NA synthesis. As yet, we do not know whether this effect is accompanied by decreased protein synthesis.

TABLE 3. EFFECT OF ANGIOTENSIN ON CATECHOLAMINE BIOSYNTHESIS IN SPLENIC NERVE

Treatment	N	Synthesis rate (nmoles/g/hr)	% Change	NA sp. act. (dis./min/μg)
None	5	11·3 ± 0·5		62·7 ± 11·3
Angiotensin (5×10^{-8} M)	5	7·1 ± 1·2*	−37	56·9 ± 8·9†
Angiotensin (5×10^{-7} M)	5	5·7 ± 1·2‡	−49	43·2 ± 9·5*

* $P < 0.05$ when compared to untreated tissue.
† Not significant, $P > 0.1$.
‡ $P < 0.01$ when compared to untreated tissue.

It is reasonably established now that acceleration of NA synthesis can occur through the depletion of a small intraneuronal pool of NA.[4,7–9] Although in some experiments we have detected a marked release of newly synthesized catechols concomitant with an acceleration of synthesis by angiotensin, this effect does not invariably occur and was not seen in the present experiments. Also in other experiments with superfused guinea pig atria no enhancement of release of exogenous ^{3}H-NA was observed in the presence of angiotensin (5×10^{-7} M).[2] We would, therefore, reject this hypothesis as the means whereby angiotensin accelerates NA synthesis, and suggest that angiotensin may exert its effect by increasing protein synthesis, possibly by influencing translation by some as yet unidentified mechanism. We are at present investigating whether the effect results in increased production of tyrosine hydroxylase and other enzymes involved in NA synthesis. The inhibitory effects of angiotensin may possibly be due to dual sites of action for the polypeptide, or may be the result of excessive concentrations of the hormone at one or another receptor site. It is also conceivable that the above observed effects on protein synthesis are not due to the presence of angiotensin itself, but rather result from fragments of angiotensin produced during the time course of the incubation.

Other results have also suggested that angiotensin may act in other systems by enhancing protein synthesis. Enhancement of aldosterone production is accompanied by increased protein synthesis in the rabbit adrenal gland[10] and these effects are blocked by puromycin. In the rat colon, enhancement of sodium and water transfer by angiotensin is also blocked by puromycin but not by actinomycin D.[11] The possibility that angiotensin may have a general role in controlling protein synthesis must now be investigated.

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Imidazole derivatives—A new class of microsomal enzyme inhibitors*

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Several groups of materials are recognized for their ability to inhibit the microsomal drug-metabolizing enzymes *in vitro* and to potentiate the action of drugs and insecticides *in vivo*. These include the classical inhibitor 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525-A) as well as other materials such as the 1,3-benzodioxoles, phenyl-2-propynyl ethers and 1,2,3-benzothiadiazoles, which have been extensively studied as a result of their activity in synergizing insecticides to insects.[1–3] As a part of our continuing search for new inhibitors of drug metabolism, we have examined the effects on rat liver microsomal enzyme activity of a series of imidazole derivatives which have previously been described as inhibitors of histidine decarboxylase.[4] The epoxidation of aldrin, the hydroxylation of dihydroisodrin and the *N*-dealkylation of *p*-chloro-*N*-methylaniline have been used as indicators of microsomal enzyme activity.

The imidazole derivatives (II–XVII) were obtained as crystalline hydrochlorides from Abbott Laboratories, North Chicago, Ill. Aldrin, dieldrin, dihydroisodrin and 6-*exo*-monohydroxydihydroisodrin were supplied by the Shell Development Co., Modesto, Calif., and were recrystallized prior to use. SKF 525-A was furnished by Smith Kline & French Laboratories, Philadelphia, Pa. *Para*-chloro-*N*-methylaniline, *p*-chloro-aniline and all biochemicals were purchased from CalBiochem, Los Angeles, Calif., and imidazole (I) was purchased from the Aldrich Chemical Co., Cedar Knolls, N.J. All other chemicals and solvents were analytical reagent grade.

Livers from male Sprague–Dawley rats, purchased from Blue Spruce Farms Inc., Altamont, N.Y., were homogenized in a Waring blender in cold 1·15% KCl (1:4, w/v) and microsomes were sedimented from the post-mitochondrial supernatant (20,000 *g* for 20 min) by centrifugation at 100,000 *g* for 1 hr. For enzyme assay, the microsomal pellet was resuspended in 1·15% KCl to a concentration of about 0·6 mg/ml. The incubation mixture and the conditions employed were identical to those previously described[5–7] as were the assays for epoxidation, hydroxylation and *N*-demethylation.[5–8] Imidazoles were added to the incubations in 10 μl water and the I_{50} values for each reaction were determined from the means of duplicate incubations with at least four inhibitor concentrations. Difference spectra were recorded with a Norelco Unicam SP-800 spectrophotometer provided with a scale expansion device and a scavenger recorder; the microsomal suspension employed for this purpose contained 1–2 mg protein/ml in 67 mM phosphate buffer, pH 7·4.

Hexobarbital sleeping time was determined in male CF_1 mice from Carworth Farms which were divided into groups at random. The animals had free access to food and water up to the time of injection. Drugs were dissolved in saline and injected intraperitoneally 0·5 hr prior to the administration of hexobarbital in saline (100 mg/kg, i.p.). Student's *t*-test was used in statistical analysis of the results.

Table 1 shows the molar I_{50} values for the imidazoles (I–XVII) against each of the three microsomal reactions investigated. Although imidazole (I) itself exhibits only slight inhibition at 10^{-4} M, all the 4(5)-substituted derivatives (II–XIV) were potent inhibitors of both epoxidation and hydroxylation,

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