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PLASMA "ANGIOTENSINASE" ACTIVITY AS A DETERMINANT OF ANGIOTENSIN PRESSOR ACTION AND TACHYPHYLAXIS IN THE RAT*

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Abstract—Dose response curves in the rat to angiotensin homologs (Des-Asp[1]-hepta-peptide and Des-Asp[1]-Arg[2]-hexapeptide) that are known to be more readily inactivated by circulating "angiotensinases" were recorded and compared with those previously published for aspartyl[1] and asparginyl[1] octapeptides. Pressor responses to equipressor doses of all four angiotensin peptides were also recorded in normal and nephrectomized rats. Plasma angiotensinase activity of adrenalectomized rats in whom a failure to develop tachyphylaxis to aspartyl[1] octapeptide is observed was compared with that of normal rats which readily demonstrate the phenomenon. Angiotensinase activity was studied as (a) per cent destruction of added aspartyl[1] octapeptide, quantified by pressor bioassay; (b) hydrolysis of aspartyl β-naphthylamide. The results show that the heptapeptide homolog (derivative) of angiotensin, unlike the asparginyl[1] octapeptide, demonstrates tachyphylaxis at biologically equivalent doses. The pressor response curves to equipressor doses of three different angiotensin peptides with different rates of inactivation *in vivo* were similar in form and duration. Plasma angiotensinase activity in adrenalectomized animals was not increased above normal at a time when tachyphylaxis was not demonstrable. The observations presented and discussed discount a principal role of plasma angiotensinase activity in the development of tachyphylaxis.

DECREASED vascular responsiveness to infused angiotensin has been noted in clinical and experimental secondary hyperaldosteronism such as hepatic cirrhosis[1] and renal ischemia.[2] Two commonly offered explanations[1, 2] for the phenomenon are (a) increase in circulating activity of plasma angiotensinase(s) and (b) the development of tachyphylaxis to the high circulating levels of endogenous angiotensin.[3] The first concept implies that diminished responsiveness results from the increased rate of degradation of the pressor peptide in plasma and consequently from the decreased number of peptide molecules reaching the receptor sites to produce the biologic effects. Conversely, tachyphylaxis has been viewed as a phenomenon of "receptor saturation"[4] by the increased number of available peptide molecules for the receptor sites, a circumstance favored by decreased activity of plasma angiotensinase(s).

We have reported recently[5] that tachyphylaxis to the pressor action of higher than minimal doses of aspartyl[1] octapeptide of angiotensin develops readily and consistently in the rat, and that the same phenomenon is not observed with asparginyl[1]

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angiotensin II (Hypertensin, CIBA). Since the asparginyl analog is degraded more readily,[6, 7] the absence of demonstrable tachyphylaxis may be attributed to this property.

The present studies were designed to test the question of whether the rate of degradation of angiotensin peptides in plasma played a predominant role in determining their pressor action and the development of tachyphylaxis. According to the hypothesis: (1) Tachyphylaxis should not occur to the pressor action of those angiotensin homologs that are destroyed in the plasma more rapidly than the asparginyl[1] octapeptide. (2) Induced lack of tachyphylaxis to aspartyl[1] angiotensin II should accompany a marked change in plasma "angiotensinase" activity. (3) Differences in the rate of peptide degradation *in vivo* should be reflected in the shape and duration of the recorded pressor curves if the circulating angiotensinases are the predominant determinant of the magnitude of drug-receptor interaction and the resulting biological response.

EXPERIMENTAL

A. Measurement of pressor response

Mean blood pressure in the carotid artery of the rat was measured directly through a pressure transducer and a strain gauge and recorded on a direct writing recorder with a paper speed of 0·5 mm/sec. The details of these methods have been described previously.[1]

Dose response curves were recorded in normal and nephrectomized rats to the following two synthetic angiotensin homologs*:

1. Des (Asp^1)-angiotensin heptapeptide
   Arg^1-Val^2-Tyr^3-Val^4-His^5-Pro^6-Phe^7;
2. Des (Asp^1)-Des (Arg^2)-angiotensin hexapeptide
   Val^1-Tyr^2-Val^3-His^4-Pro^5-Phe^6.

These two peptides represent the only two degradation products of the angiotensin octapeptide that are biologically active. The reported pressor activity of these two peptides is 40 per cent and 2 per cent, respectively, as compared with that of the parent octapeptides.[8] In the present studies, it was found to vary in different rats with a range of 20–40 for heptapeptide and ½–2 per cent for the hexapeptide.

Normal and nephrectomized rats were given alternate doses of (2·5, 5, 7·5 and 10 g) of aspartyl[1] and asparginyl[1] angiotensin octapeptides. Each dose was repeated three times. Sufficient time was allowed for each pressor response to return to the baseline. All doses were injected in a volume of less than 0·01 ml. In the same and other animals pressor responses to heptapeptide (dose range 5–50 mg) and hexapeptide (0·1–2 μg) were also recorded. All rats used in these studies weighed between 95 and 105 g.

Equipressor responses (height of pressor response in mm Hg) in the same animal to each peptide were selected and the duration of the pressor response in seconds was measured as (1) "rise time" from the start of the blood pressure rise to the peak of the pressor response, and (2) total duration from the start of blood pressure rise up to its return to the baseline.

* Supplied by CIBA, Basle, Switzerland.

B. Measurement of angiotensinase activity in rat plasma

The rate of degradation of aspartyl[1] angiotensin II, studied as described below, was accepted as an index of total angiotensinase activity. The hydrolysis of aspartyl-β-naphthylamide by rat plasma, according to the methods of Nagatsu *et al.*,[9] was taken as an additional index of the "aminopeptidase" type of angiotensinase activity. Measurement of the hydrolysis of leucyl-β-naphthylamide in rat plasma, according to the methods of Goldbarg and Rutenburg,[10] was used as an index of non-specific aminopeptidase activity. Angiotensin degrading activity was measured as follows: Freshly obtained, heparinized pooled rat plasma (enzyme source), cooled immediately to 4°, was diluted (1:5 to 1:20) with ammonium acetate buffer, pH 7·4. The concentration (1–5 μg/ml) of the added substrate (aspartyl[1] angiotensin II) was kept constant. The mixtures were incubated at 37° for 1–5 hr. At the end of the incubation period, the incubate was boiled for 2 min and centrifuged. Duplicate controls run with each set of experiments consisted of samples boiled at zero time. Supernatant from samples as well as from controls was assayed for pressor activity against standard angiotensin (Hypertensin CIBA, 1 μg/ml). A four-point bioassay was performed on rats that weighed 90–100 g and which had been nephrectomized about 16 hr earlier and treated with pentolinium tartrate (2·5 mg/100 g body wt.). The pressor responses were recorded and measured as described above. The angiotensinase activity was expressed as per cent destruction of the added octapeptide.

Bilaterally adrenalectomized rats maintained on 1% saline were studied at two time periods: (1) less than 24 hr; (2) after 96 hr (day 5) postadrenalectomy.

RESULTS

The dose response curves obtained in 4 rats with two angiotensin homologs are presented in Figs. 1 and 2. The pressor response to the heptapeptide (Fig. 1) is maximal for a dose of approximately 2·5 μg and declines at higher doses (tachyphylactic doses). This behavior of pressor response at higher doses of the heptapeptide is comparable to that of aspartyl[1] octapeptide of angiotensin reported previously[5] and shown in Fig. 2. The hexapeptide (Fig. 3), on the other hand, compares to the asparginyl[1] octapeptide of angiotensin (Fig. 4) in that the pressor response with higher doses reaches a plateau but does not decline.

The duration of the pressor response to the two octapeptides of angiotensin (aspartyl[1] and asparginyl[1]) and the two angiotensin homologs was compared at equipressor responses (see Experimental). The two octapeptides were equipressor when administered to the same animal over the range of dosage tested (2·5–10 mμg). The pressor responses shown in Table 1 ranged from 8 to 19 mm Hg in height, from 65 to 245 sec in total duration, and from 5 to 14 sec in rise time (baseline to peak). The shape and duration of pressor responses recorded in 4 different rats (2 nephrectomized) for both octapeptides did not differ significantly (mean difference in total duration, 2 sec; S.D. 30·48 to t 0·2). Equipressor doses of heptapeptide in the same rats evoked pressor response curves of shape and duration similar to those of octapeptides. The responses to hexapeptide, on the other hand, were markedly abbreviated in duration at comparable pressor dose levels. The shape and duration of pressor curves in response to all four angiotensin peptides were comparable in normal and nephrectomized rats.

Angiotensinase activity in the plasma of adrenalectomized rats was compared with

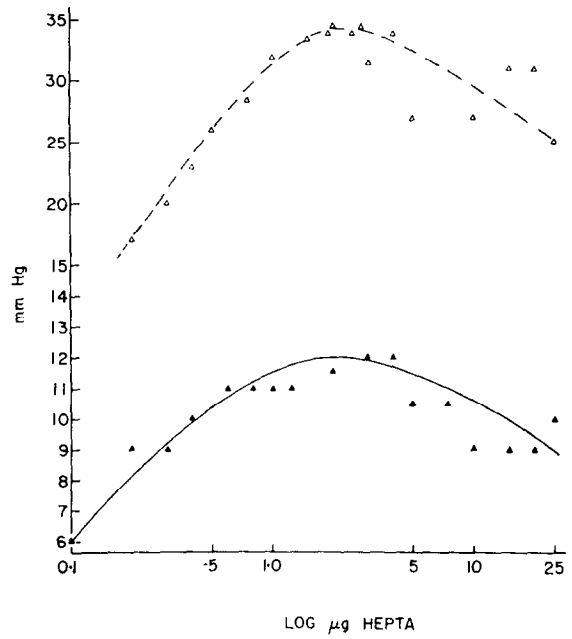

FIG. 1. Dose response curves for des-aspartyl[1]-angiotensin heptapeptide are shown in two 100-g normal rats. The ordinate (different scale for each curve) shows the change in mean blood pressure (mm Hg) after injections of peptide, and the abscissa shows the dose injected. The maximal response was achieved at a dose of about 205 μg heptapeptide, which corresponds approximately to less than 1 μg angiotensin II (octapeptide) in pressor (biological) activity. Doses of heptapeptide higher than 2·5 μg are tachyphylactic as the maximal response declines with increase in dose.

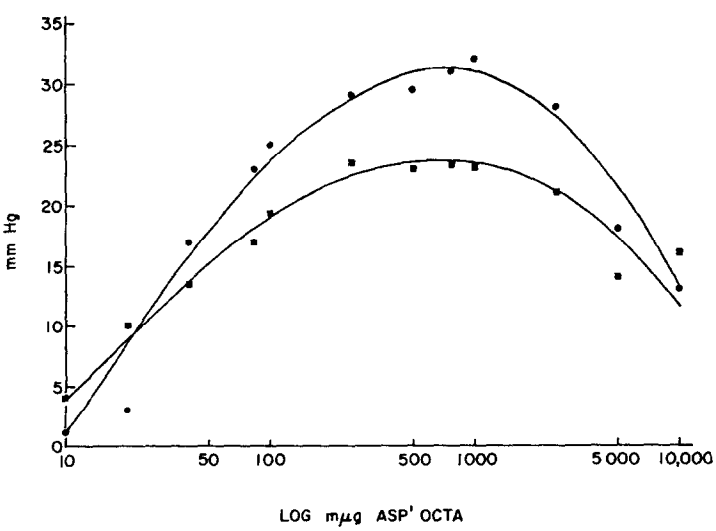

FIG. 2. Dose response curves with aspartyl[1] angiotensin octapeptide in a normal (■—■, lower curve) and a nephrectomized (●—●, upper curve) rat. The curves obtained were uniformly similar in shape in all of the animals studied; only the level of the maximal pressor response varied in different animals. (By courtesy of *Circulation Res.*).

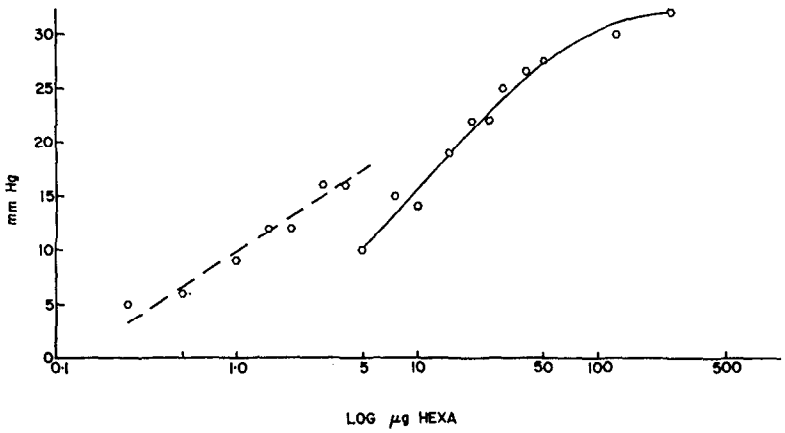

FIG. 3. Dose response curves in two normal rats (95 and 98 g) at two different dose ranges of hexapeptide of angiotensin. Ordinate shows the increase in mean arterial blood pressure (mm Hg). In order to incorporate the wide range of doses as shown and yet keep the volume of hexapeptide solution at each dose level below 0·05 ml, the injected hexapeptide was used at two different concentrations (50 g/ml and 5 mg/ml). The pressor response to hexapeptide at high doses levels off, but does not decline.

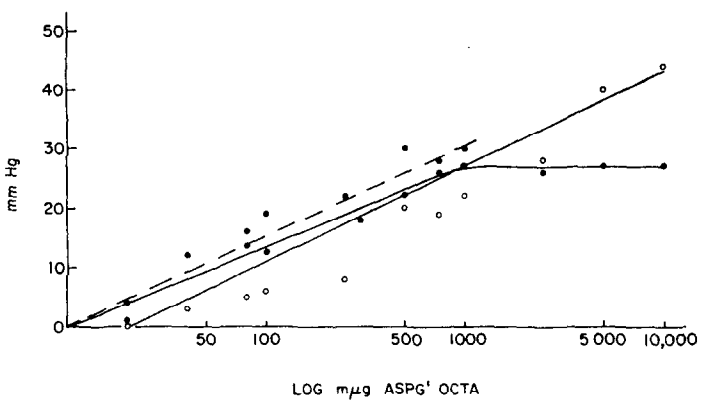

FIG. 4. Dose response curves with asparginyl[1] angiotensin octapeptide in one normal (■—■) and two nephrectomized rats (●—●). In some animals the response at higher doses levels off; it does not decline. (By courtesy of *Circulation Res.*).

that of plasma from normal rats daily up to 5 days after adrenalectomy. On the first day the plasma hydrolysis of the two chromogenic substrates was markedly depressed in the adrenalectomized as compared with the normal animals (Fig. 5). These findings were taken to indicate an overall depression of proteolytic activity. On the fifth day (96 hr after adrenalectomy), however, the hydrolysis of both of the chromogenic substrates in the plasma of adrenalectomized animals had returned to normal levels. Plasma angiotensinase activity was therefore studied in detail on the fifth postadrenalectomy day. Pooled plasma from 7 adrenalectomized rats was compared with the plasma pooled from 7 normal rats. Enzyme activities were examined in a range of dilution of the plasma (from 1 in 5 to 1 in 20) and each dilution was incubated for 1, 3 and 5 hr (Figs. 6 and 7). Hydrolysis of aspartyl β-napthylamide shown in Fig. 6 was almost identical in the two groups. The degradation rate of aspartyl[1] angiotensin

II (Fig. 7) in the plasma from adrenalectomized rats did not differ significantly from, and certainly was no greater than that in the normal plasma.

TABLE 1. PRESSOR RESPONSES TO EQUIPRESSOR DOSES OF ASPARTYL[1] (ASP[1]) AND ASPARGINYL[1] (ASPG[1]) ANGIOTENSIN OCTAPEPTIDES IN 2 NORMAL AND 2 NEPHRECTOMIZED RATS

Dose (ng/100g)	Nephrectomized rats: B.P. rise, mean (mm Hg)		Nephrectomized rats: Duration (sec)				Normal rats: B.P. rise (mm Hg)		Normal rats: Total duration (sec)	
			Total		Rise time					
	Aspg[1]	Asp[1]	Aspg[1]	Asp[1]	Aspg[1]	Asp[1]	Aspg[1]	Asp[1]	Aspg[1]	Asp[1]
2·5	10·5	11·0	76	89	5	5	8·5	8·0	66	70
2·5	10·5	11·0	89	76	5	5	7·5	8·0	65	70
2·5	10·0	11·5	76	76	5	6	8·0	8·0	117	75
5	13·0	13·0	102	109	7	7	10·0	10·0	87	98
5	13·5	13·0	102	102	6	7	10·5	10·0	102	107
5	13·5	13·0	89	173	7	9	9·5	10·0		
7·5	15·0	15·5	178	180	9	10	11·0	11·5	104	110
7·5	15·5	15·0	127	124	10	10	12·0	13·0	106	116
7·5	15·0	16·0	97	102	9	8				
10	18·0	18	218	196	11	13	12·0	13·5	104	126
10	19·0	17·5	236	235	13	12	13·0	13·0	117	103
10	18·0	17	245	197	14	11				

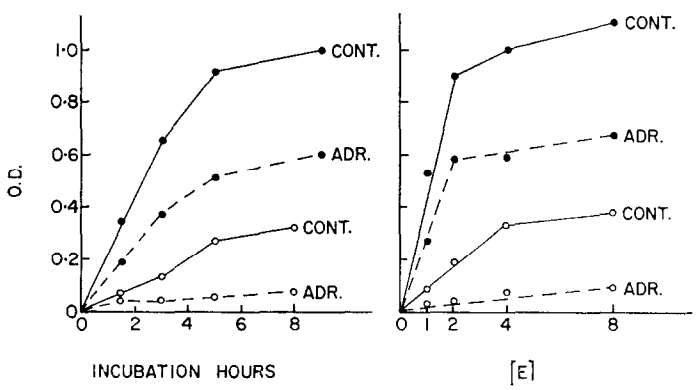

FIG. 5. Hydrolysis of two synthetic chromogenic substrates in pooled plasma from two 24-hr adrenalectomized rats (ADR) and two normal rats (cont.). Open circles indicate hydrolysis of aspartyl-β-naphthylamide and the closed circles that of leucyl-β-naphthylamide. The optical density (O.D.) readings on the ordinate are shown as a measure of released naphthylamide. The timed incubations shown in the left-hand panel of the graph were carried out at a plasma dilution of 1 in 4, and the incubations shown in the right-hand panel at four dilutions of plasma were carried out for 8 hr. [E] represents dilution of plasma (one unit of [E] denotes a dilution of 1 in 16).

DISCUSSION

It has been shown by several workers[6, 11, 12] that the N-terminal hydrolysis of angiotensin octapeptides in plasma is the rate-limiting step for the subsequent breakdown of the remaining molecule. Accordingly, the degradation products of the octapeptides, such as heptapeptide, are more readily destroyed. Tachyphylaxis, however, develops to large doses of angiotensin heptapeptide (Fig. 1) and not to similar doses of asparginyl[1] octapeptide.[5] The development of tachyphylaxis to the

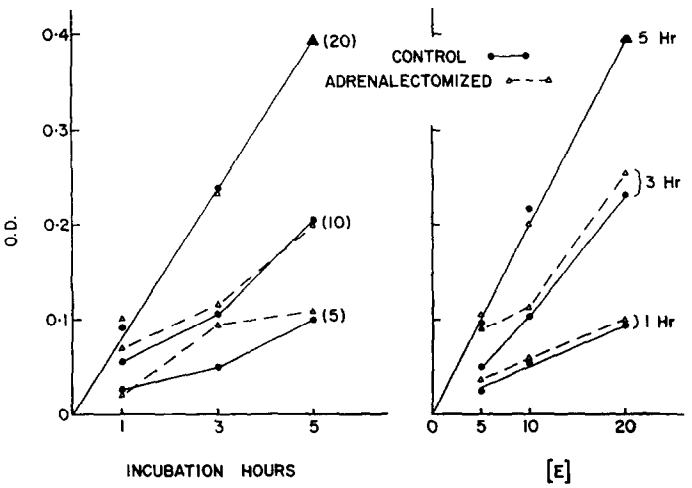

FIG. 6. Hydrolysis of aspartyl-β-naphthylamide in plasma pooled from 4 normal rats compared with plasma pooled from 4 adrenalectomized rats 5 days postadrenalectomy maintained on 1% saline. [E] denotes the per cent concentration of the pooled plasma in the incubation mixture. The right-hand panel shows the result of a 5-hr incubation at four different enzyme concentrations. O.D. readings on all samples were made against a zero time control.

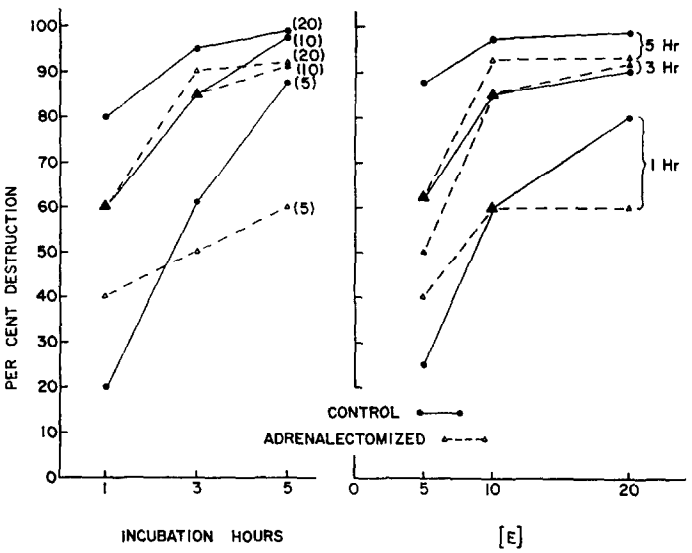

FIG. 7. Angiotensinase activity in pooled plasma from 3 normal rats (control) compared with pooled plasma from 3 adrenalectomized rats (5 days) maintained on 1% saline. The per cent destruction of added 5 μg/ml aspartyl[1] angiotensin octapeptide, detected by bioassay of the remaining amount, is shown for 1, 3 and 5 hr of incubation at 5, 10 and 20% concentration of the plasma [E].

pressor action of certain angiotensin peptides and not to that of others in the rat, therefore, cannot be explained according to the concept that the rate of plasma degradation of the peptide determines the number of the peptide molecules reaching the available receptor sites and hence their degree of saturation. The predominant

role of plasma angiotensinase activity in the development of tachyphylaxis is further discounted by the observations on adrenalectomized rats. We have previously reported that, although tachyphylaxis to a large dose of aspartyl[1] angiotensin II develops consistently in normal rats[5] (Fig. 2), the phenomenon could not be demonstrated 48–96 hr post-adrenalectomy,[13] as shown in Fig. 8. This finding was further confirmed by demonstrating in 5-day adrenalectomized rats a well maintained pressor response to

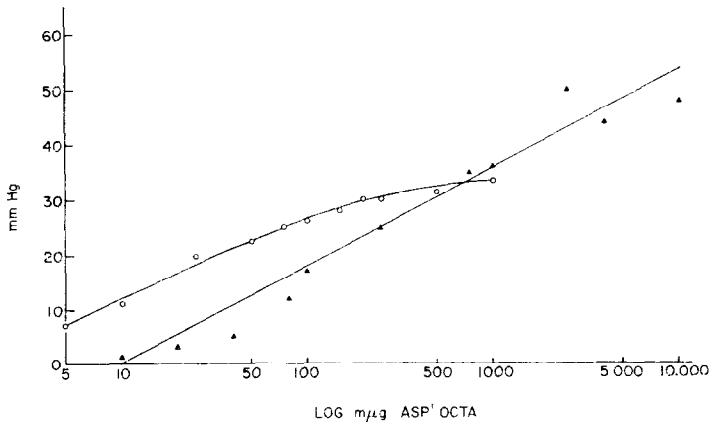

FIG. 8. Dose response curves with aspartyl[1] angiotensin octapeptide in 2 adrenalectomized rats. The open circles indicate the response in a rat 48 hr after adrenalectomy and the closed triangles show the typical pressor response obtained in rats 5 days postadrenalectomy (cf. Fig. 2).

continuous infusions of aspartyl[1] angiotensin II in doses that were consistently tachyphylactic in normal rats (unpublished observations). This failure to develop tachyphylaxis occurs without concomitant changes in plasma angiotensinase activity (Figs. 6 and 7). Furthermore, our present and previous[5] studies show that nephrectomized and normal rats exhibit parallel dose response curves to the tachyphylaxis-producing angiotensin peptides. The tachyphylactic dose of angiotensin peptides remains the same after nephrectomy, even though nephrectomized rats show a decline in angiotensin-degrading enzyme activity,[6] which Regoli *et al.*[6] have attributed to an endopeptidase of renal origin. The observations discussed, however, do not discount the possibility suggested by the work *in vitro* of Khairallah *et al.*[14] that angiotensinase may be involved in the late process of reversal of an already developed tachyphylaxis.

The degradation rate of peptides in plasma also does not appear to be the principal factor limiting their pressor action at the receptor sites. Equipressor responses to both octapeptides and the heptapeptide of angiotensin were comparable in shape and duration of the response even though these peptides show different rates of degradation *in vitro*. Recent studies of Doyle *et al.*[15] lend further support to the above observations by showing that certain angiotensin analogs (β-aspartyl angiotensin II and phenylthiocarbamyl derivative of angiotensin II) that are much less readily inactivated by plasma angiotensinases, as compared with the natural α-aspartyl angiotensin II, nevertheless show parallel dose response curves in the dog and exhibit pressor responses of similar form and duration. The same is true when nephrectomized rats are compared with normal rats (see above).

The available evidence would suggest that changes that lead to the development of angiotensin tachyphylaxis, a dose-dependent phenomenon, occur primarily at the receptor site and are not inherent in the fate of the circulating peptide molecule. Differences in biological activity of aspartyl[1] and asparginyl[1] octapeptides in isolated tissues have been described.[16, 17] Once angiotensin tachyphylaxis developed, cross tachyphylaxis can be shown to exist to all other biologically active analogs.[5, 15] It is to be noted that only those angiotensin peptides caused tachyphylaxis in the rat that have a charged *N*-terminal amino acid (aspartyl[1] octapeptide and arginyl[1] heptapeptide) and the peptides that failed to produce tachyphylaxis (asparginyl[1] octapeptide and valinyl[1] hexapeptide) have neutral amino acids as *N*-terminals. Angiotensin tachyphylaxis may therefore be viewed to result from the interaction of a large number of charged substrate molecules with the specific receptor proteins that undergo a reversible conformational change not conducive to further interaction. Such conformational perturbations are conceived to results from alteration in location of charged groups of the receptor protein, causing repulsion or displacement of water molecules and thus altering the optimal distance between reactive groups.[18–20] *N*-terminal charge of the angiotensin peptides thus seems to play, at least in the rat, a critical role in the development of tachyphylaxis quite apart from its susceptibility to inactivation by circulating angiotensinases.

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