Angiotensin Tachyphylaxis and its Reversal

By Philip A. Khairallah, M.D., Irvine H. Page, M.D., F. Merlin Bumpus, Ph.D., and R. Kazim Türker, M.D.

ABSTRACT

Tachyphylaxis to angiotensin and some analogues has been demonstrated on spirally cut arterial strips from cat, dog, sheep and rat, and on venous strips from rabbit and cat. Rabbit and guinea pig arteries do not appear to become tachyphylactic. A free C-terminal carboxyl group of angiotensin is necessary for binding with receptor sites and development of tachyphylaxis. Tachyphylaxis seems to represent saturation of receptor sites. It can be reversed by plasma fractions rich in angiotensinase A, possibly by metabolizing the N-terminal part of angiotensin directly from the bound state. Dowex 50 can also reverse it, probably by physical adsorption and stronger binding of angiotensin. Angiotensinase A does not metabolize \( \beta \)-aspartyl\(^{-1} \)-angiotensin and does not reverse tachyphylaxis to this peptide. A possible scheme of interaction between peptide and receptor site is presented.

ADDITIONAL KEY WORDS receptor sites to angiotensin saturation of receptor sites reversal of saturation angiotensinase A reversal by Dowex 50 lack of effect of catecholamines and adrenergic blocking agents cats, dogs, rabbits, rats, sheep, guinea pigs

The word tachyphylaxis was used in 1911 by Champy and Gley\(^{1} \) to describe the phenomenon of rapid immunization. Tachyphylaxis to renin was first shown in the pharmacological sense in 1898 by Tigerstedt and Bergman\(^{2} \) as diminution of pressor response following repeated injections of crude renal extracts. Page and Helmer\(^{3} \) in 1940 found the same true for natural angiotensin. Tachyphylaxis occurred after repeated injections of angiotensin, but more slowly than with renin; the mechanism of tachyphylaxis to both renin and angiotensin was interrelated. Cross tachyphylaxis was also demonstrated. They concluded that this diminution in response was due either to exhaustion of renin substrate and/or to development of inhibitors. Using pure angiotensin amide, Bock and Gross\(^{4} \) and Têtreault\(^{5} \) found a gradual decrease in pressor response after repeated injections into rats of 0.01 to 1.0 \( \mu \)g/kg. This self inhibition was demonstrated in perfused isolated hind limbs and kidneys of rats.\(^{6} \) Tachyphylaxis to angiotensin has been demonstrated in isolated rabbit heart,\(^{7} \) superior cervical ganglion of cats,\(^{8} \) guinea pig innervated vas deferens, innervated spleen of cats,\(^{9} \) perfused iris-ciliary body and enucleated whole eyes,\(^{10} \) adrenal medulla,\(^{11} \) isolated dog coronary arteries\(^{12} \) and isolated perfused human umbilical arteries.\(^{18} \)

Ditler et al.\(^{14} \) believe the action of angiotensin on vascular smooth muscle is an indirect one, mediated by liberation of norepinephrine from sympathetic nerve endings in vascular walls. Angiotensin is ineffective if the stores of norepinephrine are depleted by repeated exposure to the peptide. After partial replenishment of exogenous norepinephrine, angiotensin regains its activity. However, Supak et al.\(^{15} \) have shown that there is no block of pressor action of angiotensin by \( \alpha \)-adrenergic blocking agents such as Hydergine,\(^* \) dibenamine and tolazoline.

While studying sodium fluxes in vascular smooth muscle we observed that carotid ar-

\*Methanesulfonate derivatives of dihydroergotoxine alkaloids.
partially insensitive to angiotensin administered by superfusion. Also, spirally cut carotid arteries from dogs and cats after one contractile response to angiotensin did not respond to a second dose, in contrast to aortic strips from rabbits.10

Methods

Spirally cut carotid arteries from cats, dogs, rabbits and sheep; aortas from cats, rabbits, guinea pigs and rats; and inferior venae cavae from rabbits, cats and rats were prepared according to Furchgott et al.17 Dogs and cats were anesthetized with sodium pentothal and killed by air embolism. Rabbits, guinea pigs and rats were stunned by a blow on the head and decapitated. Blood vessels were removed within five minutes, placed in ice-cold Krebs solution, washed free of blood, and an approximate 20° spiral was cut free hand. The strips were mounted in a 25 ml muscle bath containing Krebs solution (6.9 g NaCl, 2.1 g NaHCO₃, 0.35 g KCl, 0.28 g CaCl₂, 0.11 g MgCl₂, 0.14 g Na₂HPO₄, and 2.0 g glucose per liter) at 37°, oxygenated with 95% O₂, 5% CO₂. The isotonic lever had a magnification of 4.8 times, and supplied a tension of 0.6 g. The strips were equilibrated for one to two hours, during which time most of them relaxed, almost doubling in length to 6 to 8 cm. Occasionally, a few strips were seen that did not relax, or that contracted. These strips were discarded since they would not respond to any stimulus.17

All contractile responses were expressed in percent of maximal response, so that strips could be compared to one another. Maximal contractions of a strip corresponded to a shortening of 25 to 30% of its resting length. When expressed as percentage of maximal shortening, results varied by ±5%. Stimulating agents were added every fifteen minutes, remained for five minutes; the bath was washed out twice, and this was followed by a ten-minute rest period, leading to a noncumulative dose response curve. In reversal studies 1 ml of fresh whole human plasma or soluble plasma fraction after 50% saturation with ammonium sulfate was incubated with strips for ten minutes. Angiotensinase A,18 was present in this soluble fraction. Dog, cat and rabbit plasma contained the same enzyme. The enzyme was purified further by chromatography on Sephadex G-200, equilibrated with 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer in 0.5 M NaCl, adjusted to pH 8.0. The column was eluted by a gradient produced by adding, to the Tris buffer in a mixing chamber, a solution containing 30 g of NaCl and 5 g NaHCO₃ per liter and adjusted to pH 8.10. With a column 48 cm high and a diameter of 3 cm, the enzyme appeared between the 150 and 220 ml effluents.

Also used in reversal of tachyphylaxis studies was a cation exchange resin, Dowex AG 50 W-X-2, 100-200 mesh. This was washed with 0.1 M HCl until no yellow color appeared in the effluent, followed by distilled water until the effluent reached neutrality. Washing was continued with 0.1 M CaCl₂. The effluent first turned acid, and once the resin was fully equilibrated, it returned to neutrality. The final washing was done with distilled water. One ml of thick slurry of Ca Dowex 50 was used, after final equilibration with Krebs solution. Both plasma fractions and Dowex, were added to the bath for five minutes, the bath washed twice, followed by a ten-minute rest period.

Analogues of angiotensin studied were asparagine₁-angiotensin (angiotensin amide), aspartic acid₁-angiotensin (angiotensin), β-aspartic acid₁-angiotensin (β-analogue, and asparagine₁-phenylalanine amide₁-angiotensin, (angiotensin diamide).

Results

1) Sensitivity and Tachyphylaxis in Different Species

The responses to angiotensin amide of spirally cut arteries of cats, dogs, rabbits, rats, sheep and guinea pigs were compared. Cat carotid artery was the most sensitive, responding to 0.5 to 1.0 ng/ml given as a single injection (table 1). Dog and rat arteries were least sensitive, responding to 100 to 120 ng/ml. Sensitivities to angiotensin and β-analogue were approximately the same, arteries of cats being more sensitive than those of dogs. To produce comparable contractions 20,000 times as much angiotensin diamide was required. One-half ng angiotensin amide/ml produced the same response as 10 μg of angiotensin diamide/ml. Tachyphylaxis developed in arteries from all animals examined, with the exception of those from rabbit and guinea pig which showed none to either angiotensin or its analogues in repeated doses as high as 10 μg/ml. Cross tachyphylaxis occurred among all of the angiotensin analogues that caused contractions. Cat veins were usually more responsive than arteries. One-tenth ng/ml contracted cat inferior vena cava to approximately the same degree as 1.0 ng/ml contracted cat carotid artery. These venous strips, how-
TABLE 1

Sensitivity of Artery Strips to Angiotensin

<table>
<thead>
<tr>
<th>Artery Strips</th>
<th>Angiotensin (ng/ml)</th>
<th>Contractile Response (mm ind SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat carotid</td>
<td>1.0</td>
<td>19.0 ± 1.0 (6)</td>
</tr>
<tr>
<td>Rabbit aorta</td>
<td>10.0</td>
<td>19.3 ± 3.2 (7)*</td>
</tr>
<tr>
<td>Dog carotid</td>
<td>100</td>
<td>20.2 ± 4.3 (5)</td>
</tr>
<tr>
<td>Sheep carotid</td>
<td>75</td>
<td>16.0 (2)</td>
</tr>
<tr>
<td>Rat aorta</td>
<td>120</td>
<td>12.0 (2)</td>
</tr>
<tr>
<td>Guinea pig aorta</td>
<td>50</td>
<td>23.5 (2)</td>
</tr>
<tr>
<td>Cat inferior vena cava</td>
<td>0.1</td>
<td>25 (2)</td>
</tr>
<tr>
<td>Rabbit inferior vena cava</td>
<td>10</td>
<td>34 (3)</td>
</tr>
</tbody>
</table>

*Numerals in parentheses indicate numbers of experiments.

ever, cannot be used for assay because they develop tachyphylaxis rapidly. Even though tachyphylaxis was not produced in rabbit and guinea pig arterial strips, preparations of veins showed rapid onset of tachyphylaxis.

2) DOSE RESPONSE CURVES IN DOG, CAT AND RABBIT

Increasing amounts of angiotensin amide were given every 15 minutes to obtain a dose response curve. In 27 experiments there was an increase in response of cat carotid artery up to a concentration of 2 ng/ml (fig. 1, solid line); further increase in peptide concentration led to decreases in response, so that contraction at 80 ng/ml was only one-half that at 0.5 ng/ml. Ten dog carotid arteries showed the same type of dose-response curve, except that maximum response occurred at 30 ng/ml and then decreased. Two sheep carotid arteries exhibited a maximum at 10 ng/ml and four rat aortas at 40 ng/ml.

Two guinea pig and 30 rabbit aortas (fig. 2) differed in that the response increased regularly until the muscle contracted maximally. This occurred at 50.0 ng/ml, beyond which increasing the concentration of angiotensin gave no further increase in response.

Tachyphylaxis seen in cat and dog arteries, was prevented by addition of 1 to 2 ml plasma to the 25 ml Krebs solution in the muscle bath just as was found in tachyphylactic rabbit ear vessels. In cat carotid artery strips, as seen in figure 1 (broken line), the addition of 1 ml plasma changed the dose-response curve into one similar to that of the rabbit. The strip was first made tachyphylactic to
angiotensin amide, 1 ml plasma added, and then a large dose of angiotensin amide given 15 minutes later. The response was measured and recorded. The next dose of angiotensin amide again showed tachyphylaxis, and another volume of plasma had to be added before the next point on the curve could be obtained. Something, present in plasma, reversed or prevented tachyphylaxis from developing.

Figure 2 shows also a dose-response curve of a rabbit aorta after the rabbit was infused intravenously with angiotensin amide (200 ng/kg/min) for one hour. There is almost no difference between the curve produced by aorta from infused rabbits and that from normal rabbits. Figure 3 shows a similar experiment on cat carotid arteries. One was removed before infusion of 200 ng/kg/min of angiotensin amide and the contralateral one after one hour of infusion. There was much less response by the artery taken after infusion than by the one taken before. This decreased response could also be reversed with plasma.

3) REVERSAL OF TACHYPHYLAXIS BY PLASMA FRACTIONS

Figure 1 (broken line) shows reversal of tachyphylaxis to angiotensin by plasma. The plasma fraction obtained from Sephadex G-200 column between the 150 and 220 ml effluents and containing angiotensinase A, was found to reverse tachyphylaxis. Paper electrophoresis, at pH 8.6, with barbital buffer, ionic strength 0.075, showed that over 90% of the protein in the enzyme fraction was serum albumin; the rest was chiefly α-globulin. Human albumin (Cutter, salt poor), which was dialyzed against distilled water to remove buffer, and crystalline bovine albumin (Cal Biochem) contained no angiotensinase A activity and did not reverse tachyphylaxis.

It has been suggested that angiotensinase A contains two different aminopeptidases.22 Our preparations of angiotensinase A destroyed angiotensin amide at a faster rate than angiotensin. Heating this protein fraction to 60°C for 30 minutes, or shaking with an equal volume of chloroform, completely destroyed the enzyme that metabolizes angiotensin, but had no effect on the one metabolizing angiotensin amide. Complete cross-tachyphylaxis existed between angiotensin and angiotensin amide. Tachyphylaxis of an artery to angiotensin amide was reversed with either the unheated or the heated plasma fraction, while tachyphylaxis to angiotensin was reversed only with the unheated plasma fraction. Boiling the plasma fraction destroyed both enzymatic activities, and boiled plasma did not reverse tachyphylaxis to either angiotensin or angiotensin amide.

Tachyphylaxis to the β-analogue was easily established, and showed cross reactions to angiotensin amide and angiotensin. However, this could not be reversed by plasma angiotensinase A fractions, but could be reversed by Dowex 50 (see below). It had been shown previously21 that angiotensinase A was an aminopeptidase specific for an N-terminal α-aspartic acid, and thus would not destroy the β-analogue.

4) EFFECT OF OTHER ENZYMES

Carboxypeptidase, 2.5 mg, treated with di-isopropyl fluorophosphate* (DFP) to inhibit any chymotryptic activity, did not reverse tachyphylaxis, although when incubated with angiotensin, it destroyed biological activity. On the other hand, crude leucine aminopeptidase...
Angiotensin Tachyphylaxis

Tachyphylaxis, prepared from hog kidney, reversed tachyphylaxis to both angiotensin and angiotensin amide, but not to the β-analogue. Trypsin and chymotrypsin were used also, but since, at the end of 15 minutes incubation, the muscle strip became unresponsive to serotonin, norepinephrine, vasopressin and acetylcholine, no conclusions could be drawn.

Reversal of Tachyphylaxis by Cation Exchange Resin

Dowex 50 adsorbs angiotensin from solution and binds it strongly. One ml of a thick slurry which bound 20 ng angiotensin amide, added to a 25-ml muscle bath, reversed tachyphylaxis of a cat carotid artery strip to both angiotensin and angiotensin amide. Calcium Dowex 50 was used so that no calcium was adsorbed from Krebs solution in the muscle bath, to alter contractility by changing Ca²⁺ concentration. (Fig. 4.)

Angiotensinases in Blood Vessels

Cat and rabbit aortas and their major branches were compared for content of angiotensinase. The enzyme in blood vessel is similar to that in plasma. The arteries were washed free of blood, weighed, cut into small pieces, ground with sand, and extracted about 30 minutes with 0.9% NaCl. This extract was adjusted to pH 7.5 and the angiotensinase measured by its ability to destroy angiotensin amide. Rabbit blood vessels contained about five to six times the enzymatic activity per unit wet weight as cat vessels.

Effect of Catecholamines and Adrenergic Blocking Agents on Tachyphylaxis to Angiotensin

Cat carotid strips were made tachyphylactic to angiotensin amide or to angiotensin, and norepinephrine (100 to 500 ng/ml) or epinephrine (100 to 1000 ng/ml) added, and kept in contact with the strips for 15 minutes, as described by Distler et al. No reversal of tachyphylaxis occurred in any of 25 experiments. Also, if angiotensin contracts vascular smooth muscle by liberation of norepinephrine, α and β adrenergic blocking agents should modify it, but we found that phentolamine (Regitine), in doses of 1 to 10 μg/ml, which completely blocked the contractile effect of norepinephrine and epinephrine, and with the latter, elicited relaxation, did not modify the response to angiotensin. Neither did it modify the onset or reversal, spontaneous or with plasma, of tachyphylaxis. Dichloroisoproterenol (1 μg/ml) potentiated slightly the contractile effect of norepinephrine and blocked the relaxation following epinephrine. It did not modify the response to angiotensin, or the onset or reversal of tachyphylaxis.

Miscellaneous

When a tachyphylactic cat vascular strip remained in well oxygenated Krebs solution for 1.5 to 2 hours, tachyphylaxis reversed spontaneously. Dog and sheep strips showed no reversal after 2 hours and only partial reversal after 3 hours.

Temperature has little effect on development of tachyphylaxis. There is little difference in onset, in experiments done at 22°C or 38°C. Reversal of tachyphylaxis was somewhat more rapid at 38°C than 22°C. Anesthesia had no effect, because blood vessels removed from animals under sodium pentothal or ether anesthesia, or from animals killed by decapitation or air embolism, were all reactive.

Discussion

In discussing the phenomenon of unresponsiveness it is important to distinguish between
tachyphylaxis and refractiveness. Page and Bumpus\textsuperscript{28} have considered "tachyphylaxis the result of repeated doses of a substance filling the receptor site so that no further stimulation can occur. Refractiveness, in contrast, which can occur without the administration of any drug, is spontaneous, and may change the susceptibility of the animal over short, or long, periods of time." Sometimes it is very difficult to distinguish between these two. Page\textsuperscript{24} first described refractiveness in dogs following trauma to the central nervous system and severe hemorrhage. Recently Peart reviewed other cases\textsuperscript{25} including metabolic acidosis, alteration of external sodium environment, both in vitro and in vivo, directly by experimental manipulations, or indirectly following hyperaldosteronism. Reduced responsiveness to angiotensin occurs also during pregnancy. These results have one thing in common, namely, that the reduced response to angiotensin is present initially and thus can be distinguished from tachyphylaxis.

Tachyphylaxis to angiotensin amide was first reported by Gross and Bock,\textsuperscript{4} as contrasted with that from natural angiotensin reported by Page and Helmer\textsuperscript{8} in 1940. The latter also studied the reversal by plasma, and suggested that plasma contributed an angiotensin activator, necessary to elicit contraction by angiotensin. They suggested that the activator was present in blood vessel walls for the first responses, and that this was used up in the contractile response and had to be replenished by addition of plasma. Tachyphylaxis to tyramine and ephedrine has been well studied and, according to Burn and Rand,\textsuperscript{20} is due to depletion of norepinephrine stores in vascular smooth muscle. Distler et al.\textsuperscript{10} state that angiotensin tachyphylaxis can be explained in a similar fashion.

In this paper we use the definition given by Page and Bumpus\textsuperscript{28} as a working hypothesis, namely, that tachyphylaxis is the result of repeated doses of a substance filling the receptor site so that no further stimulation can occur.

The binding of angiotensin on its receptor site does not depend significantly upon structural changes in the N-terminal end of the molecule. The three analogues tested, angiotensin, angiotensin amide and the $\beta$-aspartyl analogue, produced equivalent responses when the doses were the same, and all produced tachyphylaxis in the same dose range, contrasting with angiotensin diamide, which is required in 20,000 times higher concentration. When tachyphylaxis is produced, the latter showed cross inhibition. A free carboxyl at the C-terminal end of angiotensin is thus necessary for binding to receptor sites.

The binding of angiotensin might cause an alteration in the cell membrane, which can be considered either as a physical deformation of the membrane proteins, or as a wave of depolarization. Either may possibly elicit changes in ion fluxes, leading to muscle contraction. The peptide, however, may remain attached to its receptor site. Once all receptor sites are saturated with angiotensin, the muscle strip may be said to have become tachyphylactic.

If this saturation phenomenon exists, then angiotensin molecules must be removed by metabolism or adsorption on a stronger binding agent. Since carboxypeptidase does not restore responsiveness to tachyphylaxis, this may also suggest that the C-terminal end of angiotensin is bound. Angiotensinase A can cause reversal in about 10 to 15 minutes and if the N-terminal is free, this reaction could possibly occur in the adsorbed state, and the peptide need not be metabolized to individual amino acids before it falls away from the receptor site. Dowex 50, on the other hand, can remove angiotensin in toto by physical adsorption.\textsuperscript{21}

This idea of reversal by enzymic degradation may also explain why rabbit strips do not become tachyphylactic. Since they have about five times as much angiotensinase activity as cat aorta, the bound angiotensin may be metabolized at a faster rate, and more angiotensin would be required finally to saturate the receptor sites. Another possibility is that rabbit smooth muscle cells may contain more receptor sites. Perfused rabbit ear vessels, on the other hand, become tachyphylactic easily.\textsuperscript{8} A possible explanation is the
demonstration that rabbit veins can become tachyphylactic after repeated exposures to angiotensin. Thus, changes occurring in a perfused vascular bed could be due to saturation of receptors in either arterial or venous smooth muscle.

A sequence of reactions based on Paton's theory of drug-receptor combination\textsuperscript{27} can be postulated (fig. 5). Under usual conditions $K_2$ is very small since it requires 90 minutes or more for reversal of tachyphylaxis. However, $K_2$ can be modified by addition of angiotensinase A or Dowex 50, as shown by the decreased time needed for reversal of tachyphylaxis. $K_2/K_1 = K_{eq}$; this equilibrium constant is defined by Paton and is small. Thus, $1/K_{eq}$ is very large. According to Paton, $1/K_{eq}$ is proportional to affinity between drug and receptor; thus angiotensin must have high affinity for its receptor. Also, the rate theory predicts that unless $K_2$ is large, all drugs will show some degree of tachyphylaxis. This is well demonstrated by angiotensin. The smaller $K_2$ is, the easier will be the development of tachyphylaxis. In rabbit and guinea pig arteries $K_2$ is probably large and thus those strips will not develop tachyphylaxis. Paton also states that stimulant action is proportional to the rate of association. This is shown by comparing the effects of angiotensin amide and angiotensin diame; $K_1$ for the former must be greater than $K_1$ for the latter.

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