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Fate of angiotensin I in the toad *Bufo melanostictus*

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Summary

1. The effects of angiotensin I and II on the blood pressure of pithed toads and the disappearance of angiotensin I and II in the perfused organs of the toad were studied.

2. Angiotensin I was relatively inactive on the blood pressure of pithed toads ; it exhibited less than 3% of the pressor activity of angiotensin II.

3. Angiotensin I was not converted to angiotensin II during passage through the lungs. There was also no evidence of net conversion during passage through the kidney and hind quarters.

4. During passage through the lungs, 33–50% of angiotensin I was removed and 25–50% was removed during passage through the hind quarters. No loss of activity was detected when angiotensin II passed through the kidneys.

5. Angiotensin II passed through the lungs and kidneys without loss but 25–50% disappeared during passage through the hind quarters.

6. The relatively low pressor activity of angiotensin I together with its lack of conversion to angiotensin II in isolated perfused organs suggest that the converting enzyme is absent in the toad, *Bufo melanostictus*.

Introduction

Angiotensin exists in two naturally occurring forms: the decapeptide angiotensin I and the octapeptide angiotensin II (Skeggs, Marsh, Kahn & Shumway, 1954). Angiotensin I is comparatively inactive *in vitro*, but *in vivo* it produces a rapid and equipotent pressor response similar in time-course to that produced by angiotensin II (Skeggs, Kahn & Shumway, 1956). This increase in the activity of angiotensin I has been attributed to its rapid conversion *in vivo* to the pressor octapeptide angiotensin II.

Skeggs *et al.* (1956) isolated and purified the converting enzyme from horse plasma. Ng & Vane (1967, 1968) later showed that the plasma converting enzyme was relatively inactive and that angiotensin I was converted to angiotensin II during passage through the pulmonary circulation. Other organs inactivated both angiotensin I and angiotensin II (Ng & Vane, 1967, 1968).

The conversion of angiotensin I to angiotensin II in the lungs has been studied and defined in certain mammals, particularly the dog (Ng & Vane, 1967, 1968 ; Bakhle, 1968 ; Cushman & Cheung, 1969 ; Oparil, Sanders & Haber, 1970 ; Huggins, Corcoran, Gordon, Henry & John, 1970). However, no evidence has been presented to show whether angiotensin I is converted to angiotensin II in the pulmonary circulation of lower vertebrates. The structure of angiotensin in the lower vertebrates is not known. The following experiments were designed to study whether the mammalian Ileu^5-angiotensin I was converted to angiotensin II in the toad, *Bufo melanostictus*.

Methods

Blood pressure

Toads (40–70 g) of either sex were pithed. The abdominal skin was cut along the mid-line. The xiphisternum, coracoid and clavicle bones were removed. The anterior abdominal vein was exposed through a slit between the recti abdominis and cannulated for intravenous injections. Drugs were injected in volumes of 0·05–0·1 ml followed by 0·05 ml of saline (0·6% NaCl, w/v). The left systemic aortic arch was cannulated distal to the origin of its pulmonary artery with fine polyethylene tubing filled with heparinized saline ; the blood pressure was recorded with a Statham transducer (P23BC) on a Grass polygraph (Model 5D).

Perfusion of isolated organs

Lungs, kidneys and hind quarters were isolated and perfused with Krebs solution. The conversion of angiotensin I to angiotensin II was studied according to the method described by Bakhle, Reynard & Vane (1969). The lungs were perfused through the pulmonary arteries by a cannula inserted in the truncus arteriosus via the ventricle ; the carotid, systemic aortic arches and the cutaneous branches of the pulmo-cutaneous arches were ligated. The kidneys were perfused through the dorsal aorta. This was cannulated via the left systemic arch ; the right systemic arch, coeliac-mesenteric artery and the aorta distal to the renal arteries were ligated. The hind quarters were perfused through a cannula inserted in the dorsal aorta proximal to the origins of the common iliac arteries.

After all visible traces of blood were washed out, the organs were removed and suspended in a polypropylene chamber. They were perfused with Krebs solution at room temperature (26° C) at a rate of 2 to 4 ml/min ; the perfusion pressure was 15 to 20 mmHg (1 mmHg≡1·333 mbar). The Krebs solution was gassed with a mixture of 95% oxygen and 5% carbon dioxide. The perfusate which dripped directly over the assay organs was mixed with another stream of Krebs solution maintained at 37° C and delivered at 2 to 4 ml/min over the assay organs. Thus, the total flow rate of fluid over the assay organs was 4 to 8 ml/minute. Angiotensin solutions were infused at a rate of 0·1 to 0·4 ml/min for 2 to 5 min into the arterial cannula. The responses of the assay organs were calibrated by making infusions at a known rate into the effluent before it was mixed with the second stream of Krebs solution.

To increase the specificity of the assay organs for angiotensin II, a mixture of antagonists consisting of methysergide bimaleate (200 ng/ml), phenoxybenzamine hydrochloride (230 ng/ml), hyoscine hydrobromide (100 ng/ml), and mepyramine maleate (140 ng/ml) was infused at the rate of 0·1 ml/min into the Krebs solution after it had passed through the perfused organs (Aiken & Vane, 1970).

Bioassay for angiotensin II

The assay organs were a rat colon (Regoli & Vane, 1964) and a rat stomach strip (Vane, 1957). In some experiments, a chick rectum was included in the assay system to detect prostaglandins (Ferreira & Vane, 1967). In none of these experiments were prostaglandins detected in the perfusate. The rat colon and the rat stomach strip were used because they are about twenty times more sensitive to angiotensin II than to angiotensin I (Ng & Vane, 1967, 1968 ; Bakhle, Reynard &

Vane, 1969 ; Aiken & Vane, 1970). The load on the assay organs was 1 to 3 g and the isotonic contractions were recorded by Harvard Heart/Smooth Muscle transducers (Model 356) on a Grass polygraph.

The metabolism of angiotensins in the isolated organs was studied as follows: infusions were made into the arterial cannula and their effects on the assay organs were compared with those produced by infusions made directly over the assay organs (Bakhle *et al.*, 1969 ; Aiken & Vane, 1970). All infusions were given for 2 to 5 min until the contractions reached a plateau. At the end of the experiments, black ink was injected into the arterial cannula to check the efficiency of perfusion.

The composition of the Krebs solution was (mM): NaCl, 120 ; KCl, 4·7 ; $CaCl_2$, 2·5 ; KH_2PO_4, 1·2 ; $MgSO_4$, 1·2 ; $NaHCO_3$, 25 ; glucose, 5·6.

Drugs

Drugs used were Val^5-angiotensin II amide (Hypertensin, CIBA), $Ileu^5$-angiotensin I (gift from Dr. Wilkinson), prostaglandins E_1, E_2 and $F_{1\alpha}$ (Upjohn), methysergide bimaleate (Sandoz), phenoxybenzamine hydrochloride (SKF), hyoscine hydrobromide (E. Merck) and mepyramine maleate (May & Baker). The angiotensin I sample has 50 to 60% of the pressor activity of angiotensin in rats. This relative potency is similar to that previously reported (Ng & Vane, 1970).

All doses or concentrations are expressed in terms of the form given above.

Results

Pressor effects of angiotensin I and II

The blood pressure of ten pithed toads ranged from 5 to 40 mmHg (mean ± S.E., 20 ± 0·8). Intravenous injections of angiotensin II (0·1 to 2 μg/kg) and angiotensin I

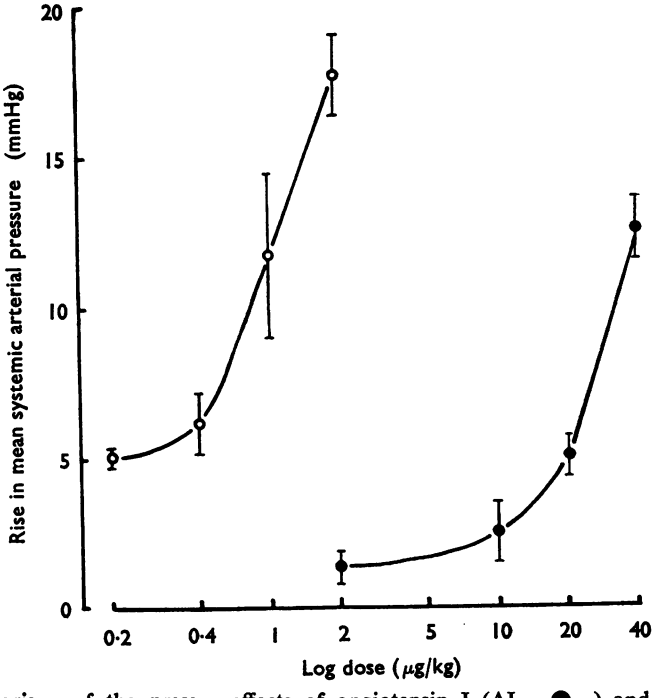

FIG. 1. Comparison of the pressor effects of angiotensin I (AI —●—) and angiotensin II (AII —○—) in ten toads. Each point represents the mean of three to six observations ; the vertical lines represent one standard error.

(2 to 40 μg/kg) produced a dose-dependent pressor response. With intervals of 4–5 min between injections, tachyphylaxis was not observed. The threshold dose for angiotensin II was 5 ng, and that for angiotensin I was 100 ng. However, the dose-response curves for angiotensin II and angiotensin I were similar in shape (Figure 1). Calculation of the relative potency along the linear portions of the dose-response curves shows that angiotensin I had less than 3% of the pressor activity of angiotensin II.

A comparison of the pressor effects of angiotensin I and II is shown in Figure 2. Saline injected in a volume of 0·1 ml produced a negligible pressor effect. Injection of angiotensin I (10 ng) failed to evoke a pressor response whereas angiotensin II (10 ng) produced a small rise in blood pressure. The pressor effect produced by 10 ng of angiotensin II could not be matched even by 100 ng of angiotensin I. On the other hand, 100 ng of angotensin II produced a marked rise in blood pressure. Had there been conversion *in vivo*, angiotensin I (100 ng) would have produced a pressor effect equivalent to that produced by half the amount of angiotensin II (50 ng), assuming that the angiotensin I was only 50% pure.

FIG. 2. Pressor effects of angiotensin I (AI) and angiotensin II (AII) in a pithed toad (female, 50 g). AI (10 ng, 100 ng) failed to evoke a pressor response, whereas AII (10 ng) caused a small increase and AII (100 ng) caused a marked increase in blood pressure. Time, 1 minute; vertical scale, mmHg.

Experiments with isolated perfused organs

The results are summarized in Table 1. Angiotensin II passed through the toad perfused lungs without loss. The results were similar to those obtained in other studies (Goffinet & Mulrow, 1963; Hodge, Ng & Vane, 1967; Biron, Meyer & Panisset, 1968; Leary & Ledingham, 1969; Bakhle *et al.*, 1969; Aiken & Vane, 1970). The isolated perfused lungs of mammals convert 8 to 50% of angiotensin I

TABLE 1. *Percentage disappearance of angiotensin I (AI) and angiotensin II (AII) after intra-arterial infusions into isolated perfused organs of the toad*

Organ		Angiotensin concentration (ng/ml)	Percentage disappearance Range	Mean±S.E.	Experiments
Lungs	AI	40–80	33–50	40±0·6	7
	AII	0·4–4	No loss detected		5
Kidneys	AI	70–800	No loss detected		5
	AII	2–9	No loss detected		7
Hind quarters	AI	80–160	25–50	38±0·7	7
	AII	2–8	25–60	41±0·5	8

Figures for the disappearance were calculated by comparison of the contractions of the rat colon and rat stomach strip produced by intra-arterial infusions with those produced by infusions made directly over the assay organs.

to angiotensin II (Bakhle *et al.*, 1969 ; Aiken & Vane, 1970). In the present experiments, however, no conversion was detected in the lungs of the toads. In contrast, 33 to 50% (mean ± S.E., 40 ± 0·6) of angiotensin I disappeared during passage through the lungs. Figure 3 shows an experiment in which angiotensin I was preferentially removed whereas angiotensin II passed through the lungs without loss.

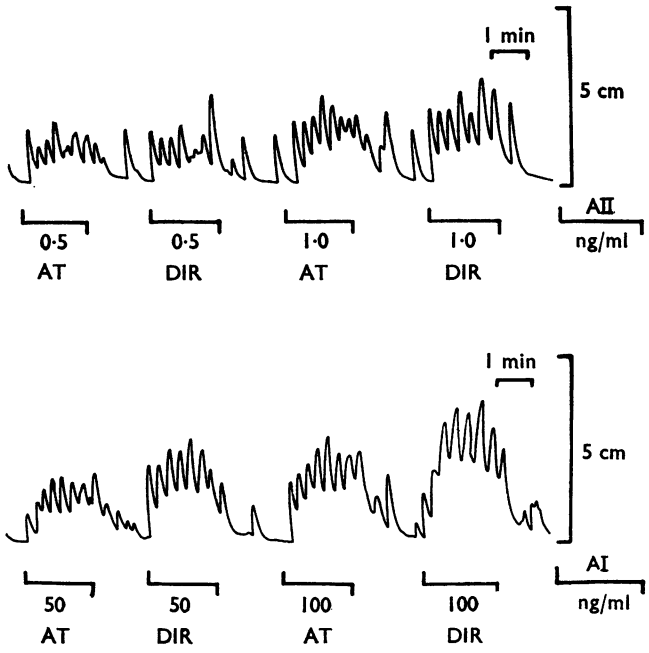

FIG. 3. A pair of toad lungs were perfused with Krebs solution at 2 ml/minute. The effluent superfused a rat colon, the contractions of which to angiotensin II (AII) and angiotensin I (AI) are shown. Infusions given into the pulmonary artery are indicated by AT ; infusions given directly over the tissue are indicated by DIR. The top tracing shows that AII (0·5 and 1·0 ng/ml) given either AT or DIR produced identical effects on the rat colon ; this showed that AII passed through the lungs without being removed. The bottom tracing shows that AI (100 ng/ml) given through the lungs (AT) produced an effect similar to that produced by AI (50 ng/ml) given directly (DIR) over the rat colon ; this showed a 50% disappearance of AI on transit through the lungs. Time, 1 min ; vertical scale, 5 cm.

Angiotensin I was inactive on the blood vessels of the hind legs of the frog, *Rana pipiens* (Carlini, Picarelli & Prado, 1958), but it exhibited more than 50% of the vasoconstrictor activity of angiotensin II on the hind legs of the toads, *Bufo itericus* (Carlini *et al.*, 1958) and *Bufo arenarum* (Halvorsen, Fasciolo, Calvo, Puebla, Binia, Alonzo & Fernandez, 1958). The marked vasoconstrictor activity of angiotensin I in these preparations suggests that angiotensin I may be converted to angiotensin II in the toad hind legs.

The present perfusion experiments could not measure the *in situ* conversion of angiotensin I to angiotensin II in the hind legs of *Bufo melanostictus*. Assay of the perfusate provided a measure of residual angiotensin I or excess angiotensin II which escaped inactivation in the perfused organ. Since intra-arterial angiotensin I (80 to 160 ng/ml) always produced a smaller effect on the assay organs than similar concentrations of angiotensin I given into the effluent, it was assumed that there was no net synthesis of angiotensin II, but that there was disappearance or inactivation of angiotensin I in the perfused hind legs. The disappearance of angiotensin I was

25 to 50% (mean ± S.E., 38 ± 0·7); for angiotensin II, it was also 25 to 50% (mean ± S.E., 41 ± 0·5). These results agree with those obtained *in vivo* (Hodge *et al.*, 1967; Biron *et al.*, 1968) and those *in vitro* (Bakhle *et al.*, 1969).

The perfused toad kidneys did not remove angiotensin I or angiotensin II in contrast to the 90 to 95% removal of both peptides by the isolated kidneys of the dog and cat (Bakhle *et al.*, 1969). The lack of inactivation of angiotensins by toad kidneys was not due to insufficient perfusion, since the kidney sections were well stained by ink at the end of the experiments. The isolated kidneys of the rat did not remove angiotensin II but 30% of angiotensin I was inactivated (Bakhle *et al.*, 1969). These variations in the metabolism of angiotensins by isolated kidneys may be explained by the difference in species or by the non-physiological state of the perfused tissues.

Discussion

The relatively low pressor activity (1 to 3%) of angiotensin I in toads agreed qualitatively with the lack of vasoconstrictor effect of angiotensin I on the isolated perfused kidneys of the rat (Skeggs *et al.*, 1956). It was also similar to the low pharmacological activity (2 to 10%) of angiotensin I on some isolated smooth muscle preparations (Ng & Vane, 1968; Aiken & Vane, 1970). Since the high pressor activity of angiotensin I in mammals is due to its conversion to angiotensin II (Ng & Vane, 1967, 1968, 1970; Biron & Huggins, 1968; Oparil *et al.*, 1970), it is conceivable that the mammalian Ileu^5-angiotensin I was not converted to angiotensin II in the toad, *Bufo melanostictus*.

The lack of conversion of angiotensin I to angiotensin II in the toad was substantiated by experiments on perfused organs. Isolated toad lungs perfused with Krebs solution did not convert angiotensin I to angiotensin II. There was also no evidence for net conversion in the perfused kidneys or the hind limbs. These *in vitro* results, coupled with the relatively low pressor activity of angiotensin *in vivo*, lead to the conclusion that the mammalian type of converting enzyme is absent in the toad, *Bufo melanostictus*.

Angiotensin I was removed by the toad perfused lungs and lower limbs, but not by the perfused kidneys. Angiotensin II passed through the perfused lungs and kidneys without loss. Both angiotensin I and angiotensin II were removed to a similar extent in the toad hind limbs. The mechanism for the removal of these angiotensins in the intact toad or perfused organs is not known at present. Although angiotensinase is present in toad plasma (Bean, 1942), tissue peptidase are primarily responsible for the inactivation of angiotensins *in vivo* (Ng & Vane, 1967, 1968; Biron *et al.*, 1968).

In mammals, two enzymes are involved in the biosynthesis of angiotensin II. The first enzyme is renin which is secreted from the kidney (Tigerstedt & Bergmann, 1898); the second or converting enzymes is located primarily in the lung (Ng & Vane, 1967). Renin acts on a substrate in the plasma protein to produce the relatively inactive angiotensin I (Skeggs *et al.*, 1956) and the converting enzyme in the lung generates from angiotensin I the physiologically active angiotensin II (Ng & Vane, 1967, 1968). Recently, converting enzyme activity has also been reported in the kidneys of the dog (Oparil *et al.*, 1970; Franklin, Peach & Gilmore, 1970).

The existence of a renin-angiotensin system in amphibians has not been conclu-

sively established. First, renin seems to be absent in the kidneys of the toad, *Bufo arenarum* (Bean, 1942), but juxtaglomerular type cells have been described in the frog (McManus, 1942) and renin has been extracted from the kidneys of the frog, *Rana catesbeiana* (Johnston, Davis, Wright & Howards, 1967). Second, angiotensin I was inactive on the blood vessels of the hind legs of the frog, *Rana pipiens* (Carlini *et al.*, 1958), but it was active on those of the toad, *Bufo itericus* (Carlini *et al.*, 1958) and *Bufo arenarum* (Halvorsen *et al.*, 1958).

Although the presence of renin has not been detected in the kidney of the toad, *Bufo melanostictus*, the comparatively small pressor effect of angiotensin I on the blood pressure suggests that it is unlikely to be converted to angiotensin II *in vivo*. This result coupled with its lack of conversion to angiotensin II during passage through the perfused organs strongly suggests that the converting enzyme and perhaps the renin-angiotensin system of the mammalian type has not emerged in the phylogenetic development of the toad, *Bufo melanostictus*.

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