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Role of the endothelium on the facilitatory effects of angiotensin I and angiotensin II on noradrenergic transmission in the caudal artery of the rat

D.F. Story & J. Ziogas[1]

Department of Pharmacology, University of Melbourne, Parkville, Victoria, 3052, Australia

1 Perfusion of carbogen gas through the lumen of the rat caudal artery abolished the dilator response to acetylcholine (1 μmol l^{-1}) in artery segments which had been precontracted with noradrenaline (50 nmol l^{-1}). Hence, it was assumed that gas perfusion was effective in removing the vascular endothelium.

2 Angiotensin I (30 nmol l^{-1}) and angiotensin II (10 nmol l^{-1}) enhanced the responses of artery segments to field stimulation of their sympathetic nerves (0.5 Hz, 10 s). In arteries with an intact endothelium the ability of each peptide to enhance responses to stimulation was the same whether applied through the lumen or to the adventitial surface.

3 Removal of the endothelium, by gas perfusion, did not significantly alter the facilitatory effects of extraluminally or intraluminally applied angiotensin I or angiotensin II.

4 The converting enzyme inhibitor enalaprilat was equally effective in inhibiting the facilitatory effect of angiotensin I in the presence and absence of an intact endothelium.

5 It is concluded that in the rat caudal artery, conversion of angiotensin I to angiotensin II does not depend on an intact endothelium and that the facilitatory effect of angiotensin II on noradrenergic neuroeffector transmission is not modified by, or dependent on, an intact endothelium.

Introduction

It is generally accepted that the major site for the formation of angiotensin II from its decapeptide precursor, angiotensin I, is the pulmonary vasculature (Ng & Vane, 1967; Aiken & Vane, 1970). However, conversion of angiotensin I to angiotensin II has also been shown to occur in other tissues including kidney (Franklin *et al.*, 1970), brain (Ganten *et al.*, 1976), heart (Cross *et al.*, 1981; Nakashima *et al.*, 1982) and several extrapulmonary blood vessels (DiSalvo & Montefusco, 1971; Malik & Nasjletti, 1976; Ljung *et al.*, 1981).

We have also published evidence of extrapulmonary conversion of angiotensin I to angiotensin II in that angiotensin I was found to enhance sympathetic neuroeffector transmission in isolated preparations of guinea-pig atria and rat caudal artery. In both tissues the effect of angiotensin I but not angiotensin II was blocked by angiotensin converting enzyme inhibitors (Ziogas *et al.*, 1984a,b).

In the pulmonary vasculature angiotensin converting enzyme has been shown to be associated with the plasma membrane of endothelial cells (Ryan *et al.*, 1975). However, in extrapulmonary blood vessels, there is evidence that converting enzyme activity may be present in other cell types. Thus, immunofluorescent microscopy has identified converting enzyme activity in both the intima and adventitia of small arteries in the guinea-pig kidney (Horovitz, 1981), and Velletri & Bean (1982) found angiotensin converting enzyme activity in homogenates of the rat aortic tunica media and adventitia but not in homogenates of the aortic intima. Saye *et al.* (1984), demonstrated that in rabbit aorta the endothelium influenced the rate of conversion of angiotensin I to angiotensin II, but that the endothelium was not essential for the conversion.

The endothelium of blood vessels may release susbtances which profoundly influence the reactivity of the medial smooth muscle to drugs and endogenous substances. Thus, it has been known for some time that the vascular endothelium is a source of prostaglandins (Moncada *et al.*, 1977) and more recently Furchgott (Furchgott & Zawadzki, 1980; review, Furchgott, 1983) found that the vasodilator effects of acetylcholine and of a number of other substances is

[1]To whom all correspondence should be addressed

dependent upon the release of an endothelium-derived relaxing factor (EDRF). Although there is no evidence to suggest that the angiotensin peptides release EDRF, in isolated vascular preparations angiotensin II has been shown to release prostaglandins which may oppose the vasoconstrictor action of the peptide (Webb, 1982).

In view of the uncertainty about the role of endothelial cells in the conversion of angiotensin I to angiotensin II in extrapulmonary blood vessels and the recognition that the endothelium is a source of substances which may mediate or modify the effects of vasoactive drugs, we have investigated the consequence of removal of the endothelium on the interaction of both angiotensin I and angiotensin II with sympathetic neuroeffector transmission in the caudal artery of the rat.

Methods

Caudal artery preparations

Wistar rats (300–450 g) of either sex were killed by decapitation and a 3 to 4 cm proximal segment of the central tail artery was carefully dissected from each rat. The segments were cannulated at each end under warm Krebs-Henseleit solution continuously bubbled with 5% CO_2 plus 95% O_2 (carbogen). The preparations were tested for leakage and those which were satisfactory were set up for perfusion in one of two ways.

For both perfusion techniques the artery segments were mounted vertically, proximal end lowermost. Two platinum electrodes, 3 mm apart, were placed around each segment just above the proximal cannula to permit field stimulation of the adventitial sympathetic nerves. The lumen of each artery segment was perfused by directing Krebs-Henseleit solution at a constant rate of 4 ml min^{-1} through the lower cannula. In some experiments the perfusate passing from the upper cannula was allowed to superfuse the adventitial surface of the artery (perfused and superfused preparations). In other experiments the cannulated artery segments were placed in a jacketed organ bath containing 10 ml of Krebs-Henseleit solution. In these experiments the perfusate passing from the upper cannula was not allowed to mix with the solution in the organ bath bathing the adventitial surface of the artery segment (perfused and bathed preparations). With both procedures the luminal perfusion pressure was monitored using a Statham P23Db pressure tranducer and a Rikadenki (R-O2) potentiometric recorder.

After allowing artery preparations to perfuse for 20 min they were tested for the presence of funtional endothelial cells. Firstly, vasoconstriction was produced by introducing noradrenaline (50 nmol l^{-1}) into the luminal perfusate and after the perfusion pressure had stabilized at an elevated level (2–3 min) acetylcholine (1 μmol l^{-1}) was also added to the perfusion fluid (see Figure 2). A dilator response to acetylcholine was taken to indicate the presence of functional endothelial cells (Furchgott, 1983). Preparations which failed to respond initially to acetylcholine with a dilatation were discarded. The test procedure was repeated several times during the course of each experiment.

In preparations which were perfused and separately bathed the effects of either angiotensin I or angiotensin II on vasoconstrictor responses to sympathetic stimulation were determined firstly for extraluminal application of the test peptide (added to the solution in the organ bath), and secondly for intraluminal application (introduced into the perfusion fluid). In preparations which were perfused and superfused the effects of angiotensin I on the responses to sympathetic stimulation were determined by adding the peptide to the perfusion-superfusion solution. Where the effect of angiotensin I was determined in the presence of the converting enzyme inhibitor enalaprilat (MK-422), the inhibitor was added to the perfusion-superfusion fluid and was then present for the remainder of the experiment.

In each case stimulation-evoked vasoconstrictor responses, measured as increases in perfusion pressure, were elicited at 90 s intervals with 10 s trains of square wave pulses (0.2 ms) applied at a frequency of 0.5 Hz. The stimulation voltage was supraximal at about 15 V. The effects of angiotensin I or angiotensin II on the stimulation-evoked responses were determined by comparing the mean of 4 to 6 responses obtained immediately before addition of the test peptide with the mean of 4 to 6 responses obtained in the presence of the peptide.

Removal of endothelium

The vascular endothelium was removed by the technique of Spokas & Folco (1984). This involved interrupting the luminal perfusion and passing a stream of carbogen gas through the lumen for 90 s at a pressure of 40 mmHg. Subsequent loss of a dilator response to acetylcholine (see above) was taken as evidence of loss of endothelial cell function.

Physiological solution

The composition (in mmol l^{-1}) of the Krebs-Henseleit solution was: NaCl 118, KCl 4.7, $NaHCO_3$ 2.5, KH_2PO_4 1.03, $MgSO_4 7H_2O$ 0.45, $CaCl_2$ 2.5, (+)-glucose 11.1 and disodium ethylenediamine tetraacetic acid 0.067.

The Krebs-Henseleit solution in the perfusate reservoirs and in the organ bath (perfused and separate-

ly bathed preparations) was maintained at 37°C and bubbled continuously with carbogen gas.

Drugs

The drugs used were: angiotensin I (Sigma), [Val^5]-angiotensin II (Ciba), noradrenaline bitartrate (Sigma), acetylcholine perchlorate (Sigma) and enalaprilat (MK422; the active metabolite of enalapril) (Merck).

All stock solutions were made up with Krebs-Henseleit solution, except enalaprilat, which was first dissolved in methanol and subsequently diluted with Krebs-Henseleit solution.

Statistical analysis

Where pairing of experimental observations was appropriate, data was analysed statistically by 2-tailed paired Student's *t* tests. The linear portions (20 to 80% of maximum effect) of log concentration-response relationships were established by least squares regression. The individual lines so obtained were tested for linearity (unpaired *t* test) and for the significance of the linear correlation. Paired log concentration-response lines were compared by testing for significant departures from parallelism and coincidence (unpaired *t* tests). Dose-ratios for the inhibitor effect of the converting enzyme inhibitor enalaprilat on the responses to angiotensin I were determined as the antilogarithm of the horizontal displacement of the angiotenin I log concentration-response line produced by the inhibitor. In all statistical tests significant differences were accepted for probability values less than 0.05.

Results

Comparison of the effects of extraluminal and intraluminal administration of angiotensin I and angiotensin II

In artery preparations with functional endothelium, in which the lumen was perfused and the adventitial surface separately bathed, the mean control vasoconstrictor response to stimulation of the adventitial sympathetic nerves was 56.2 mmHg (s.e.mean, = 6.5, $n = 27$). Angiotensin I or angiotensin II when added either to the solution perfusing the lumen (intraluminal administration) or to the solution bathing the adventitial surface (extraluminal administration) enhanced the stimulation-evoked responses. Figure 1 shows the mean increase in stimulation-evoked responses produced by angiotensin I (30 nmol l^{-1}) and angiotensin II (10 nmol l^{-1}) for each route of administration. For both peptides there was no significant

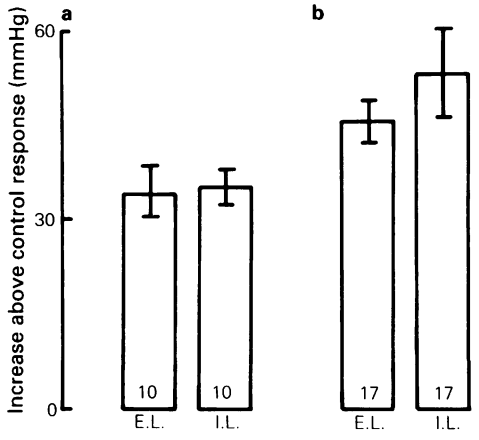

Figure 1 Effects of extraluminal (E.L.) and intraluminal (I.L.) application of (a) angiotensin I (30 nmol l^{-1}) and (b) angiotensin II (10 nmol l^{-1}) on vasoconstrictor responses of segments of rat caudal artery to stimulation of the adventitial sympathetic nerves (10 s periods at 0.5 Hz). Each column shows the mean, and the vertical bar the s.e.mean of the increase above the control responses to stimulation. The mean control vasoconstrictor response of all preparations in the absence of the peptides was 56.2 mmHg (s.e.mean = 6.5, $n = 27$). The value of n is shown in each column.

difference ($P > 0.20$, paired *t* tests) between the enhancements observed with the two routes of administration: for angiotensin I the mean difference between the enhancement produced by extraluminal and intraluminal administration was − 0.2 mmHg (s.e.d. = 4.8, $n = 10$) and for angiotensin II the mean difference between the enhancement produced by the two routes of administration was 5.1 mmHg (s.e.d. = 4.1, $n = 17$).

Effect of gas perfusion on responses to noradrenaline, acetylcholine and sympathetic stimulation

Figure 2a shows the effects of gas perfusion on the constrictor response to 50 nmol l^{-1} noradrenaline and on the dilator response to 1 μmol l^{-1} acetylcholine (obtained in the presence of noradrenaline), for a single perfused and separately bathed artery preparation. The pooled data, for a series of fifteen such experiments, are shown in Figure 3. The mean vasoconstrictor response to noradrenaline was not significantly altered by gas perfusion ($P > 0.10$, paired *t* test), the mean difference between the noradrenaline response before and after gas perfusion being 4.5 mmHg (s.e.d. = 4.1, $n = 15$). In contrast, the dilator response to acetylcholine was abolished by gas

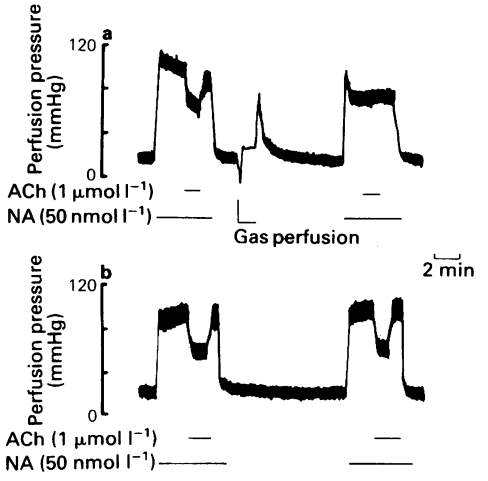

Figure 2 Effect of gas perfusion on the vasoconstrictor response to noradrenaline (NA) and on the vasodilator response to acetylcholine (ACh) in a single rat caudal artery preparation (a). (b) Shows responses to noradrenaline and acetylcholine in an artery preparation which was not subjected to gas perfusion. The periods when the drugs were present are indicated by the horizontal lines below the traces.

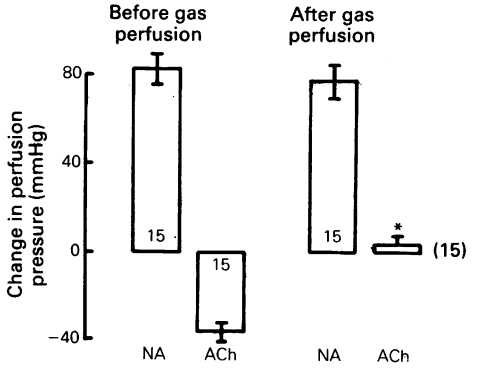

Figure 3 Effects of gas perfusion on the vasoconstrictor response of rat caudal artery preparations to noradrenaline (NA, 50 nmol l^{-1}) and on the vasodilator response of the preparations to acetylcholine (ACh, 1 μmol l^{-1}) elicited during the noradrenaline-evoked vasoconstriction. The columns show the means, and the vertical lines the s.e.mean of the responses to noradrenaline and acetylcholine (change in perfusion pressure). The value of n is shown in each column.
*Significant difference after gas perfusion ($P < 0.0005$, paired t test).

perfusion, indicating loss of functional endothelium, the mean difference before and after gas perfusion being 28.7 mmHg (s.e.d. = 3.6, n = 15). There were no significant time-dependant changes in the effects of noradrenaline or acetylcholine in twelve preparations which were not subjected to gas perfusion ($P > 0.10$, paired t tests).

Gas perfusion did not significantly alter the vasoconstrictor response to stimulation of the adventitial sympathetic nerves ($P > 0.10$, paired t test). Thus, the mean response to stimulation before gas perfusion was 56.2 mmHg (s.e.mean = 6.5, n = 15) and the mean difference between the responses before and after gassing was 2.9 mmHg (s.e.d. = 8.3, n = 15).

Effect of endothelium removal on the facilitatory effects of angiotensin I and angiotensin II

Table 1 summarizes the mean increases in the stimulation-evoked responses produced by angiotensin I (30 nmol l^{-1}) and angiotensin II (10 nmol l^{-1}) in perfused and separately bathed preparations, before and after endothelium removal. Each peptide was given both by the extraluminal route and by the intraluminal route. Loss of endothelial function, as evidenced by the loss of a dilator response to acetylcholine, had no significant effect on the mean enhancements produced by angiotensin I administered either extraluminally or intraluminally ($P > 0.10$, paired t tests). Likewise the

Table 1 Increase in vasoconstrictor responses to sympathetic stimulation

	Angiotensin I (30 nmol l^{-1})		*Angiotensin II (10 nmol l^{-1})*	
	Extraluminal application	*Intraluminal application*	*Extraluminal application*	*Intraluminal application*
Before gas perfusion	29.5 ± 8.5 (6)	29.8 ± 5.1 (6)	39.7 ± 8.7 (9)	46.0 ± 10.0 (9)
After gas perfusion	27.7 ± 6.3 (6)	21.8 ± 3.1 (6)	39.9 ± 9.7 (9)	38.0 ± 7.2 (9)

Values shown are a mean increase above control response (mmHg) ± s.e.mean. The number of experiments is indicated in parentheses.

mean enhancements observed with both extraluminal and intraluminal angiotensin II were, in each case, not significantly altered by endothelium removal ($P > 0.20$, paired t tests).

Effect of enalaprilat on the facilitatory effect of angiotensin I

The effect of the converting enzyme inhibitor enalaprilat (0.03 μmol l^{-1}) on the facilitatory effect of angiotensin I on vasoconstrictor responses to sympathetic stimulation was investigated in perfused-superfused artery preparations with functional endothelium and in preparations in which endothelial function had been destroyed by gas perfusion. Log concentration-response relationships for enhancement of stimulation-evoked vasoconstriction by angiotensin I were obtained as described by Zioges *et al.* (1984b). As shown in Figure 4 the linear portions of the log concentration-response relationships for angiotensin I obtained in the absence of enalaprilat in preparations with functional endothelium and those obtained in preparations in which endothelial function had been destroyed by gassing did not depart significantly from coincidence ($P > 0.25$, unpaired t test). In the presence of enalaprilat the log concentration-response relationship to angiotensin I was displaced to the right to the same extent in preparations in which endothelial function was present (dose-ratio = 11.8; 95% confidence limits: 6.5 to 22.5) and in preparations in which endothelial function was destroyed (dose-ratio = 9.5; 95% confidence limits: 7.0 to 12.0). Thus, the effect of inhibition of angiotensin converting enzyme on the interaction of angiotensin I with sympathetic neuroeffector transmission was independant of the presence or absence of functional endothelium.

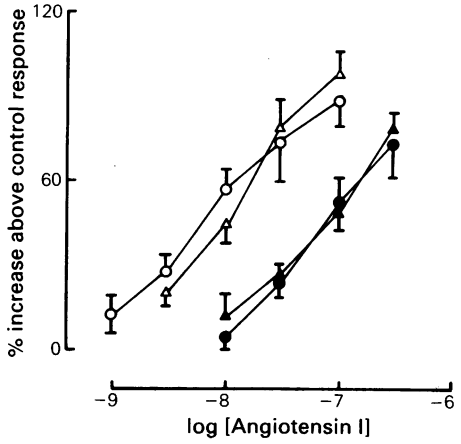

Figure 4 Effect of enalaprilat (0.03 μmol l^{-1}) on the log concentration-response relationships for the enhancement by angiotensin I of sympathetic stimulation-evoked (0.5 Hz for 10 s) vasoconstriction in rat caudal artery segments with intact endothelium (○) and in artery segments in which the endothelium had been removed by gas perfusion (△). In each case the open symbols (○,△) represent the mean enhancements of the responses produced by angiotensin I in the absence of enalaprilat and the solid symbols (●,▲) represent the mean enhancements produced by angiotensin I in the presence of enalaprilat. The points represent the mean values obained in 4 to 6 experimetns. The vertical lines indicate the s.e.mean.

Discussion

The present study was undertaken to determine if the endothelial cells of the rat caudal artery are involved in the conversion of angiotensin I to the biologically active angiotensin II, and also, whether the presence of a functional endothelium influences the facilitatory effect of angiotensin II on noradrenergic neuroeffector transmission in the artery. The interpretation of the findings depends on knowledge of the effectiveness with which the technique of interrupting the normal fluid perfusion of the artery and passing a stream of dry gas through the lumen results in a loss of functional endothelium.

Perfusion of gas through the vascular lumen to remove endothelial cells was first used *in vitro* (for the caudal artery of the rat) by Spokas & Folco (1984). Whereas these investigators demonstrated that gas perfusion abolished the ability of acetylcholine to release the endothelium-derived substance which mediates the vasodilator effect of acetylcholine (EDRF), they did not produce histological evidence of endothelial cell loss. Previously, however, Fishman *et al.* (1975) demonstrated that passing a gentle stream of air through the common carotid artery of the rat *in vivo* resulted in total loss of the endothelial cell layer of the vessel. Moreover, the technique was shown not to cause significant damage to the medial smooth muscle. It is likely that the passage of gas through the artery lumen resulted in drying of the endothelial cell layer such that on recommencing perfusion with fluid the cells are sloughed from the vessel wall. In view of the findings of Fishman *et al.* (1975) it would seem likely that in the present study the endothelial cells of the rat caudal artery were effectively removed by the gassing procedure; this is supported by the finding that the dilator effect of acetylcholine was totally abolished by gas perfusion. The advantage of this technique over abrasive procedures for removing vascular endothelium is that, apart form the brief period when perfusion with Krebs-Henseleit solution was stopped and carbogen gas passed through the lumen, no

manipulation of the artery preparation is required.

We have previously provided evidence that in the isolated caudal artery of the rat angiotensin I is rapidly converted to angiotensin II, which can subsequently facilitate responses of the vessel to sympathetic nerve stimulation (Ziogas *et al.*, 1984b). The present findings indicate that in this artery the intramural generation of angiotensin II from angiotensin I is not dependant on the presence of functional endothelial cells since the ability of angiotensin I to facilitate responses to sympathetic stimulation was not altered after gas perfusion. Furthermore, the facilitatory effect of angiotensin I was identical whether it was perfused through the artery lumen or added to the solution bathing the adventitial surface of the artery.

Angiotensin converting enzyme is a membrane bound exopeptidase that is located on the lumenal surface of pulmonary endothelial cells (Ryan *et al.*, 1975). Therefore it is possible that after gas perfusion some converting enzyme may remain either on intact endothelial cells or associated with cell membrane fragments which may remain attached to the internal elastic lamina of the artery. This, however, seems unlikely in view of the finding of Fishman *et al.* (1975) that complete removal of the endothelium layer occurs with the gas perfusion procedure. Thus, it would appear that in the caudal artery of the rat angiotensin converting enzyme is present at sites other than, or in addition to, the endothelium. The present findings are consistent with those of Saye *et al.* (1984), who found that removal of the endothelium from rabbit aorta did not inhibit the contractile response to angiotensin I, although it did slow the rate of conversion of angiotensin I to angiotensin II. In previous studies angiotensin converting enzyme activity has been demonstrated in the medial and adventitial surfaces of rat aorta (Velletri & Bean, 1982) and in cultures of vascular smooth muscle (Dzau, 1984), as well as in cultured (Johnson & Erdös, 1977) or isolated endothelial cells (Ody & Junod, 1977). The lack of consequence of endothelial removal on the facilitatory effect of angiotensin I could be due to the precursor peptide itself having a facilitatory effect on the noradrenergic transmission process. A residual, direct effect of angiotensin I on smooth muscle has been proposed (Aiken & Vane, 1970; Saye *et al.*, 1984). This possibility can, however, be rejected since the converting enzyme inhibitor enalaprilat reduced the facilitatory action of angiotensin I on sympathetic noradrenergic transmission. Furthermore, the effectiveness of enalaprilat in reducing the facilitatory effect of angiotensin I was the same in preparations with a functional endothelium and in preparations in which endothelial function had been destroyed by gas perfusion.

The second aspect of the study was to determine the extent to which the facilitatory effect of angiotensin II on noradrenergic neuroeffector transmission is influenced by, or dependent on, the vascular endothelium. It was established that the facilitation produced by angiotensin II was unchanged after removal of the endothelium, whether the peptide was given extraluminally or intraluminally. Thus, it is unlikely that in this preparation substances released from the endothelium, such as prostaglandins, modify the effect of angiotenin II as has been shown to occur in renal veins from dogs, pigs and rabbits (Webb, 1982) and in the perfused rat heart (Lanier & Malik, 1982).

In conclusion, it appears that in the caudal artery of the rat the endothelium is not necessary for the intramural generation of angiotensin II from angiotensin I, nor does the presence of a functional endothelium appear to influence the facilitatory effect of angiotenin II, on the responses of the artery to sympathetic nerve stimulation.

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