Local formation of angiotensin II in the rat aorta: effect of endothelium

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- 1 The local formation of angiotensin II (AII) from its precursors, angiotensin I (AI) and tetradecapeptide (TDP) renin substrate, was studied in intact (with endothelium) and rubbed (without endothelium) aortic rings of the rat.
- 2 AI and TDP renin substrate maximally contracted intact tissues in a similar way to AII. The same observations were made in rubbed tissues.
- 3 The maximal response and the sensitivity of the aorta to these agonists were greater in rubbed than in intact tissues.
- 4 In intact preparations, methylene blue increased the contractile response to AII and TDP to the same extent as endothelium removal.
- 5 In intact preparations, AII receptor blockade completely suppressed all contractile responses, converting enzyme inhibition completely blocked the responses to AI and TDP, and renin inhibition partially blocked the responses to TDP.
- 6 In rubbed preparations, AII receptor blockade completely inhibited all contractile responses, converting enzyme inhibition completely suppressed the responses to AI but only partially inhibited those to TDP, and renin inhibition partially blocked the responses to TDP.
- 7 In conclusion, the formation of AII from TDP and its blockade by a converting enzyme inhibitor and a renin inhibitor shows that converting enzyme and a renin-like aspartic proteinase are present in the aortic wall. Furthermore, the results show that the endothelium is not essential for the conversion of the TDP to AII, but modulates the responses to locally formed AII through the release of endothelium-derived relaxing factor (EDRF).

Introduction

There is substantial evidence that the essential components of the renin-angiotensin system (RAS) (angiotensinogen, renin, converting enzyme, angiotensin II (AII) receptors) are present in a variety of tissues including blood vessels and the heart (Desjardin-Giasson *et al.*, 1981; Re *et al.*, 1982; Campbell & Habener, 1986; Dzau, 1986; Campbell, 1987; Samani *et al.*, 1987). In particular, the endothelium of blood vessels has been shown to be a major site of conversion of AI to AII by the converting enzyme located on its luminal surface (Caldwell *et al.*, 1976; Ryan *et al.*, 1976). Moreover, bovine aortic endothelial cells have been shown to contain renin and angiotensinogen and to be capable of synthesizing and secreting angiotensins (Lilly *et al.*, 1985; Kifor & Dzau, 1987). Also AII receptors have recently been described in porcine aortic endothelial cells (Patel *et al.*, 1989). These findings indicate that endothelial cells may be an important site for the synthesis of AII in vascular tissues. AII produced locally in vascular tissues may be involved in the regulation of vascular tone, through a direct vasoconstrictor action, an ability to increase noradrenaline neuroeffector function and/or through endothelial stimulation of prostacyclin (PGI₂) and, possibly, endothelium-derived relaxing factor (EDRF) biosynthesis (Zimmerman *et al.*, 1984; Peach, 1988). Locally synthesized AII may also play an important role in the development of vascular hypertrophy (Geisterfer *et al.*, 1988).

Local formation of AII from AI and the synthetic tetradecapeptide (TDP) renin substrate has been shown in different vascular tissues (Malik & Nasjletti, 1976; Oliver & Sciacca, 1984; Ziogas *et al.*, 1986; Juul *et al.*, 1987; Eglème *et al.*, 1989a). In the rat aorta, we showed that TDP was metabolized to AII via AI (Eglème *et al.*, 1989a).

The present experiments were performed to study the role of the endothelium in the formation of AII from AI and TDP

renin substrate in the rat isolated aorta. The contraction of aortic rings with and without endothelium to AI and TDP was therefore studied in the presence and absence of inhibitors of the RAS and compared with the contraction induced by AII. A preliminary communication of these results has been presented to the British Pharmacological Society (Eglème *et al.*, 1989b).

Methods

Animals

Male Sprague-Dawley rats (RA 25, Tif, Sisseln, Switzerland) weighing 280–350 g were used. The rats were fed a regular diet and allowed free access to tap water.

Experimental protocol

The rats were killed by decapitation, and the thoracic aorta was removed and cleaned. Four rings (2 mm wide) were cut from the aorta and were left intact or mechanically rubbed to remove endothelial cells. The rings were then carefully suspended under a tension of 2 g in 20 ml organ baths filled with a physiological salt solution (mm: NaCl 112, KCl 5, NaHCO₃ 25, KH₂PO₄ 1.0, MgSO₄ 1.2, CaCl₂ 2.5 and glucose 11.2) at 37°C gassed with 95% O₂:5% CO₂ to obtain a pH of 7.4. Contractile responses were measured with an isometric strain gauge (Biegestab K30, Hugo Sachs Elektronik, FGR) coupled to a potentiometric pen recorder (Lineacorder Mark VII WR3101, Hugo Sachs Elektronik, FGR). After an equilibration period of 60 min, tissues were exposed to a depolarizing solution containing 100 mM KCl (mm: NaCl 17, KCl 100, NaHCO₃ 25, KH₂PO₄ 1.0, MgSO₄ 1.2, CaCl₂ 2.5 and glucose 11.2), which induced a maximal contraction. After 15 min of contraction, acetylcholine 1 µM was added which induced a relaxation in intact rings and no relaxation in rubbed rings. Preparations were then washed and left for a further 60 min equilibration.

Single responses to AII, AI or TDP were obtained by adding the appropriate concentration to the bath and only

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Figure 1 Concentration-effect curves for (a) angiotensin II (AII), (b) AI and (c) tetradecapeptide in rat aortic rings with (●) and without (○) endothelium. Responses are expressed in tension (mg). Each point is the mean of at least 4 observations and vertical lines show s.e.mean.

Figure 2 Time-course of the contraction induced by (a) angiotensin II ($1 \mu\text{M}$) and (b) tetradecapeptide ($1 \mu\text{M}$) in rat aortic rings without endothelium (○) and with endothelium untreated (●) or pretreated (▲) for 30 min with methylene blue $3 \mu\text{M}$. Responses are expressed in tension (mg). Each point is the mean of at least 4 observations.

Table 1 Effect of pharmacological inhibitors of the renin-angiotensin system on the responses induced by submaximal concentrations of angiotensin II (AII), AI and tetradecapeptide (TDP) in rat aortic rings with and without endothelium

	Responses (mg)	
	Endothelium present	Endothelium absent
AII		
Control	424 ± 39 (14)	1016 ± 83 (5)
[Sar ¹ , Ile ⁸]-AII (18 nM)	0 (8)***	0 (5)***
Enalaprilat (6.1 μM)	430 ± 71 (5)	1014 ± 83 (5)
CGP 44099 (4 μM)	408 ± 52 (9)	1084 ± 99 (5)
AI		
Control	449 ± 44 (15)	940 ± 57 (5)
[Sar ¹ , Ile ⁸]-AII (18 nM)	0 (8)***	0 (5)***
Enalaprilat (6.1 μM)	0 (8)***	24 ± 24 (5)***
CGP 44099A (4 μM)	387 ± 20 (7)	903 ± 88 (5)
TDP		
Control	343 ± 18 (17)	940 ± 65 (5)
[Sar ¹ , Ile ⁸]-AII (18 nM)	0 (8)***	0 (5)***
Enalaprilat (6.1 μM)	18 ± 12 (6)***	198 ± 87 (5)***
CGP 44099A (4 μM)	175 ± 36 (9)***	280 ± 55 (5)***

Values shown are means $\pm$ s.e.mean and numbers in parentheses represent number of observations.

Significant differences between control and treated preparations were determined by unpaired Student's *t* test:

****P* < 0.001.

one contraction per ring was performed, to avoid the problems of tachyphylaxis observed with these components of the RAS. When concentration-effect curves of an agonist were constructed, each ring of the same aorta was exposed to a different concentration of the agonist. When the effect of methylene blue or of a pharmacological inhibitor of the RAS was studied, the substance was preincubated with the ring for 30 min before the addition of one of the components of the RAS, and only one agonist per aorta was studied. Appropriate controls were run at the same time.

Drugs

AII (Hypertensin, CIBA), AI (Bachem), [Sar¹, Ile⁸]-AII (Bachem), enalaprilat (Merck) and acetylcholine HCl (Roche) were dissolved in bidistilled water as 1 mg ml^{-1} stock solutions and stored at -20°C . Equine TDP (Bachem) was dissolved in a slightly acidic solution as a 1 mM stock solution and stored at -20°C . CGP 44099A (Z-Arg-Arg-Pro-Phe-Val-Cha ψ [CHOHCH₂] Val-Val-Tyr-OMe $\cdot$ 2HCl, Ciba-Geigy) was dissolved in an aqueous solution containing 5% glucose to a 1 mg ml^{-1} stock solution and stored at -20°C . Methylene blue (Fluka) was prepared as a 10 mM stock solution in bidistilled water.

Statistical analysis

The maximal amplitudes of the contractions induced by each agonist were measured. Results are expressed as means $\pm$ s.e.mean. Significance of differences was evaluated by use of Student's *t* test, *P* values less than 0.05 being considered significant.

Results

AII, AI and TDP produced concentration-dependent increases in tension of rat aortic rings with and without endothelium, when each drug was added to the bath in single concentrations (Figure 1). In the presence of endothelium, the maximal responses induced by AII, AI and TDP were reached with a concentration of $10 \mu\text{M}$ and were of similar magnitude (AII: $496 \pm 46 \text{ mg}$, $n = 5$; AI: $530 \pm 73 \text{ mg}$, $n = 5$; TDP: $429 \pm 49 \text{ mg}$, $n = 4$). They represented about 50% of the maximal response induced by a depolarizing solution ($1079 \pm 30 \text{ mg}$, $n = 23$). The sensitivity of the aorta to TDP ($\text{EC}_{50} = 0.25 \pm 0.09 \mu\text{M}$, $n = 4$) was about half the sensitivity to AI and AII ($0.14 \pm 0.05 \mu\text{M}$ and $0.13 \pm 0.03 \mu\text{M}$, $n = 5$), but this difference was not statistically significant. In the absence of the endothelium, the maximal responses to TDP, AI and AII were obtained at a concentration of $1 \mu\text{M}$ and were twice those recorded in the presence of endothelium. They were comparable to the maximal contraction induced by the depolarizing solution ($1198 \pm 43 \text{ mg}$, $n = 16$) (Figure 1). The sensitivity of the aorta to TDP, AI and AII was also increased. This effect was, however, less pronounced for TDP (EC_{50} values were decreased by about 5 fold) than for AII and AI (EC_{50} values were decreased by about 15 and 11 fold, respectively).

To determine whether EDRF was involved in the endothelium-dependent inhibition of the contractile responses induced by TDP and AII, we examined the effects of methylene blue, an inhibitor of soluble guanylate cyclase which inhibits the action of EDRF at the level of vascular smooth muscle cells (Ignarro & Kadowitz, 1985; Al Osachie & Godfraind, 1986), on contractile responses to AII and TDP. In intact preparations, methylene blue ($3 \mu\text{M}$) increased the contractile response to TDP and AII ($1 \mu\text{M}$) to a similar extent as the endothelium removal (Figure 2). The inhibition by the endothelium of the contractile response to AII in the rat aorta could be due to either the basal release of EDRF, or stimulated release of EDRF by AII. AII did not appear to liberate EDRF since it did not relax an intact preparation of rat aorta which had been precontracted with prostaglandin $\text{F}_{2\alpha}$ ($3 \mu\text{M}$), but induced a further contraction (unpublished observation). Such conclusions confirm those of Bullock *et al.* (1986).

To ascertain whether removal of the endothelium modified the pathways of AII formation from AI and TDP in the rat aorta, specific pharmacological inhibitors of the RAS were used, and their effects were evaluated on contractions induced by a submaximal concentration of AII, AI or TDP in intact and rubbed aortic rings. This concentration was estimated to be about $1\text{ }\mu\text{M}$ in intact preparations, and 100 nM in denuded preparations. In both intact and rubbed preparations, none of the inhibitors studied had a significant effect on baseline tone when added alone. In the presence of endothelium, AII receptor blockade (with $[\text{Sar}^1, \text{Ile}^8]\text{-AII}$, 18 nM) completely inhibited the responses to AII, AI and TDP. Converting enzyme inhibition (enalaprilat, $6.1\text{ }\mu\text{M}$) had no effect on the response to AII but completely blocked the responses to AI and TDP. Renin inhibition (CGP 44099A, $4\text{ }\mu\text{M}$) had no effect on the responses to AII and AI but inhibited the responses to TDP by about 49% (Table 1). Endothelium removal did not seem to modify the main pathway of AII formation from AI and TDP. In summary, AII receptor blockade completely blocked all contractile responses, converting enzyme inhibition completely suppressed the responses to AI but only partially inhibited those to TDP, and renin inhibition blocked by about 70% the responses to TDP (Table 1).

Discussion

The present results show that removal of the endothelium in the rat aorta did not prevent the local conversion of AI and TDP renin substrate to AII. Moreover, they demonstrate that the maximal response and the sensitivity of the tissue to AII, whether given exogenously or locally formed from AI and TDP renin substrate, were increased by removal of the endothelium. Our observations concerning the influence of the endothelium on the response to exogenous AII confirm previous studies performed in the rat aorta and in some other vascular tissues, such as bovine coronary artery, rabbit mesenteric and coeliac arteries (Oshiro *et al.*, 1985; Bullock *et al.*, 1986; Gruetter *et al.*, 1988). However, in other vessels such as rabbit aorta or canine carotid artery, the endothelium has no effect on the contractile response to AII (Saye *et al.*, 1984; D'Orléans-Juste *et al.*, 1985). In the present study, methylene blue, an inhibitor of EDRF, produced the same potentiation of the contractile response to AII as endothelium removal. This indicates that, in the rat aorta, the inhibitory effect of the endothelium on the contraction induced by AII may be attributed to the release of EDRF, as has been proposed by Bullock *et al.* (1986) and Gruetter *et al.* (1988).

When the endothelium was removed, the contractile responses of the rat aorta to AI and AII were potentiated to a similar extent. Contractions induced by a submaximal concentration of AI were still completely blocked by the converting enzyme inhibitor, enalaprilat, and by the receptor antagonist, $[\text{Sar}^1, \text{Ile}^8]\text{-AII}$. These observations show that, in the rat aorta without endothelium, AI was still converted to AII by converting enzyme. This indicates that the converting enzyme was present at sites other than the endothelium, and in amounts sufficient to allow the local conversion of AI to AII. These findings do not bear out previous data indicating that the converting enzyme is mainly localized in vascular endothe-

lium (Caldwell *et al.*, 1976; Ryan *et al.*, 1976). However, they are in good agreement with the results of more recent pharmacological and biochemical studies. In different vessels, such as the rabbit aorta, the rat femoral artery, the rat caudal artery and the guinea-pig pulmonary artery the absence of endothelium does not alter the functional response to AI (Saye *et al.*, 1984; Story & Ziogas, 1986; Schölkens *et al.*, 1987; Urabe *et al.*, 1987). Moreover, it has been suggested that angiotensin-converting enzyme activity is negligible in the vascular endothelium of rat and rabbit aortae, and of the guinea-pig pulmonary artery, since it did not differ in preparations with and without endothelium (Velletri & Bean, 1982; Schölkens *et al.*, 1987).

When the endothelium was removed, or when the action of EDRF was chemically inhibited by methylene blue, the responses induced by TDP and AII ($1\text{ }\mu\text{M}$) were increased to a similar extent. These observations demonstrate that the endothelium was not the major site of TDP metabolism but modulated the action of AII formed locally from TDP. However, a minor role of the endothelium in the conversion of TDP to AI cannot be ruled out: although endothelial removal increased the maximal contractile responses to TDP and AII to a similar extent, the responses induced by the lowest concentrations were potentiated less for TDP than for AII. The results of the present experiments also indicate that the main pathway of AII formation from TDP in the rat aorta, i.e. a two-step metabolism through a renin-like enzyme and converting enzyme (Eglème *et al.*, 1989a), is not modified by removal of endothelium. In rubbed preparations, the contraction induced by a submaximal concentration of TDP was still inhibited by AII receptor antagonism, converting enzyme inhibition and renin inhibition. However, some small differences in the inhibitory effects of enalaprilat and CGP 44099A were observed when the endothelium was removed. The renin inhibitor, CGP 44099A, inhibited the response to TDP to a greater extent when the endothelium was removed (70% compared to 49% with endothelium). Removal of the endothelium may facilitate the access of this compound to its critical sites of action. In contrast, the converting enzyme inhibitor, enalaprilat, was less effective in blocking the response to TDP when the endothelium was removed. This might indicate that, when the endothelium is removed, a small amount of TDP is converted directly to AII without first being metabolized to AI. Another explanation may be that the preparation of TDP was contaminated with small amounts of AII barely detectable in the less sensitive intact aortic rings but detectable in the more sensitive rubbed rings.

In summary, we showed that removal of the endothelium potentiated the maximal contractile responses to AII, AI and synthetic TDP renin substrate to a similar extent, and did not modify the main pathway of AII formation from TDP i.e. a two-step pathway formation through a renin-like enzyme and converting enzyme. Such observations indicate that the endothelium of the rat aorta is not essential for the vascular conversion of AI and TDP to AII, but may modulate the responses of locally formed AII through EDRF release.

The authors wish to thank Dr H. Rueeger for the synthesis of the renin inhibitor, Prof. K.G. Hofbauer and Dr L. Criscione for fruitful discussions.

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(Received August 24, 1989
Accepted February 13, 1990)