British Journal of Pharmacology (1995) 114, 925–930 © 1995 Stockton Press All rights reserved 0007–1188/95 $9.00

Role of endothelin-1 and the ET_A receptor in the maintenance of deoxycorticosterone acetate-salt-induced hypertension

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1 To search for a possible role for endothelin-1 (ET-1) in deoxycorticosterone acetate (DOCA)-salt-induced hypertension, we examined changes in concentration of ET-1 in vascular and renal tissue in DOCA-salt hypertensive rats and evaluated the antihypertensive effect of the ET_A receptor antagonist, FR139317.

2 There was an increase in aortic immunoreactive-ET (IR-ET) concentrations in association with hypertension-induced treatment. There were no significant changes in ET-1 levels in the kidney with DOCA-salt treatment.

3 In DOCA-salt hypertensive rats, a significant correlation ($r = 0.83$, $P < 0.01$) was found between aortic IR-ET concentrations and systolic blood pressure.

4 High-performance liquid chromatography analysis of the aortic extract from DOCA-salt rats revealed one major component corresponding to the elution position of synthetic ET-1.

5 The intravenous bolus injection of FR139317 ($10\ mg\ kg^{-1}$) produced a slight decrease in blood pressure in the control rats and in the DOCA-salt hypertensive rat, FR139317 had a more pronounced hypotensive effect.

6 We propose that ET-1 production in vascular tissues is increased in DOCA-salt hypertensive rats. In addition, our study indicates the pathophysiological importance of increased endogenous ET-1 in the maintenance of DOCA-salt-induced hypertension, through interaction of the peptide with ET_A receptors.

Keywords: Endothelin-1 (ET-1); endothelin receptor; ET_A antagonist; deoxycorticosterone acetate-salt hypertension

Introduction

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor substance so far identified (Yanagisawa *et al.*, 1988). This peptide possesses a wide variety of biological actions (Rubanyi & Botelho, 1991) and may play a role in the various cardiovascular disorders such as cerebral vasospasm after subarachnoid haemorrhage (Matsumura *et al.*, 1991), acute renal failure (Kon & Badr 1991), heart failure (Margulies *et al.*, 1990), atherosclerosis (Lerman *et al.*, 1991) and hypertension (Vanhoutte, 1993; Lüscher *et al.*, 1993). Circulating ET-1 concentrations are increased during hypertension (Kohno *et al.*, 1991; Widimsky *et al.*, 1991). However, circulating ET-1 levels do not reflect the local production of the peptide. Indeed, the change in ET-1 content of tissues occurs without change in circulating ET-1 levels (Hughes *et al.*, 1992; Larivière *et al.*, 1993).

One study found that there was an increased vascular content of ET-1, with no change in circulating ET-1 concentrations in deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Larivière *et al.*, 1993). It has also been reported that renal ET-1 production is altered in spontaneous hypertensive rats (Kitamura *et al.*, 1989; Hughes *et al.*, 1992). Thus, these changes in local ET-1 production might participate in the development and/or the maintenance of hypertension in these models. However, the functional significance of increased or decreased local ET-1 levels in these hypertensive models remains obscure.

Several ET receptor antagonists have been developed and may be useful for evaluating physiological or pathophysiological roles of endogenous ET-1 and its receptor subtypes (Ihara *et al.*, 1992; Clozel *et al.*, 1993; Sogabe *et al.*, 1993). FR139317 ((**R**)2-[(**R**)-2-[(S)-2-[[1-hexahydro-1H-azepinyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl)propionic acid) is an ET_A receptor antagonist which inhibits ET-1-induced vasoconstrictor effects *in vitro* and *in vivo* (Sogabe *et al.*, 1993). To explore the contribution of ET-1 in DOCA-salt-induced hypertension, we examined changes in ET-1 concentrations of vascular and renal tissues in DOCA-salt hypertensive rats and evaluated the antihypertensive effect of FR139317.

Methods

DOCA-salt treatment and blood pressure measurement

Male Sprague-Dawley rats, weighing 160–180 g were anaesthetized with sodium pentobarbitone ($50\ mg\ kg^{-1}$, i.p.) and the right kidney was removed via a right flank incision. After a 1-week postsurgical recovery period, the rats were treated twice weekly with deoxycorticosterone acetate (DOCA) suspended in corn oil, which was administered subcutaneously ($15\ mg\ kg^{-1}$) and 1% NaCl added to their tap water for drinking. Control rats were uninephrecotomized but not given DOCA or salt. Systolic blood pressure was monitored with a tail cuff and a pneumatic pulse transducer. The rats were exsanguinated 1 or 4 weeks after treatment and concentrations of ET-1 were measured.

Tissue extraction and ET-1 measurement

ET-1 was extracted from the kidney, according to the method of Fujita *et al.* (1994). Briefly, kidneys were weighed and homogenized for 60 s in 8 vol ice-cold organic solution

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(chloroform/methanol, 2:1, including 1 mM N-ethylmaleimide). The homogenates were left overnight at 4°C, then 0.4 vol distilled water was added to the homogenates. In case of extraction from the aorta, thoracic aortae (4 cm) were removed from animals, rapidly cleaned of fat and adherent connective tissue, weighed and homogenized for 60 s in 4 ml ice-cold organic solution, as described above. The homogenates were left overnight at 4°C, then 0.4 ml of distilled water was added to the homogenate. Those aortic or renal homogenates were then centrifuged at 3000 r.p.m. for 30 min and the supernatant was stored. Aliquots of the supernatant were diluted 1/10 with a 0.09% trifluoroacetic acid (TFA) solution and applied to Sep-Pak C18 cartridges. The sample was eluted with 3 ml of 63.6% acetonitrile and 0.1% TFA. Eluates were dried in a centrifugal concentrator and the dried residue was reconstituted in assay buffer for radioimmunoassay (RIA). The clear solution was subjected to RIA. Recoveries of ET-1 from aorta and renal tissues in our extraction procedures were approximately 80%.

RIA for ET-1 was carried out as described elsewhere (Matsumura *et al.*, 1990b). The limit of detection of ET-1 in this assay was 3 pg/tube. ET-1 antiserum (a generous gift from Dr Marvin R. Brown, Department of Medicine, University of California, San Diego, U.S.A.) did not cross-react with big ET-1, as described (Hexum *et al.*, 1990).

Reverse-phase high-performance liquid chromatography

After application of the tissue sample to a Sep-Pak C18 cartridge, the dried residue was dissolved in 0.5 ml of 0.02% TFA instead of radioimmunoassay buffer and a 0.4 ml portion was then applied to a Capcell-Pak C18-SG300 column (4.6 × 250 mm, Shiseido, Tokyo, Japan), using a high-performance liquid chromatography (h.p.l.c.) system (model 600E, Waters Chromatography Division). Elution was performed with 0.02% TFA in water (solvent A) and 0.02% TFA in acetonitrile (solvent B). The gradient was linear from 0% to 35% v/v solvent B for 15 min, followed by isocratic elution at 35% v/v solvent B for 15 min and a linear gradient from 35% to 63% v/v solvent B for 15 min. The flow rate was 0.5 ml min^{-1}. Each fraction was evaporated and assayed for immunoreactive-ET (IR-ET) by RIA.

Effect of FR139317 (ET_A receptor antagonist)

Experiments were carried out on rats treated with DOCA-salt for 4 weeks and on age-matched control rats. The animals were anaesthetized with sodium thiobutabarbitone (Inactin, 100 mg kg^{-1}, i.p.) and placed on a heated surgical tray that maintained the rectal temperature between 37° and 38°C. After tracheotomy, the right femoral vein was cannulated for bolus injection of the drug. The right femoral artery was also cannulated for blood pressure measurement with a pressure transducer. After a 90 min equilibration period, FR139317 (10 mg kg^{-1}) or vehicle was administered intravenously by slow bolus injection (2 min). The doses of FR139317 used in this study have been shown to produce complete inhibition of ET-1-induced pressor action (Sogabe *et al.*, 1993). Blood pressure was recorded continuously on a polygraph (Nihon Koden, RM 6000G, Tokyo, Japan).

Statistical analysis

All values are expressed as mean ± s.e.mean and were analyzed statistically by an unpaired *t* test. Aortic IR-ET levels were correlated with systolic blood pressure by linear regression analysis. $P<0.05$ was considered significant.

Drugs

FR139317 was a kind gift from Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. FR139317 was dissolved in 1 N NaOH and diluted with saline. Other chemicals were purchased from Nacalai Tesque, Inc (Kyoto, Japan).

Results

Table 1 summarizes the comparative data on groups of animals treated with DOCA-salt for 7 or 28 days and of age-matched controls. There were no differences in systolic blood pressure of the control animals and those treated with DOCA-salt for 7 days. After 28 days of DOCA-salt treatment, systolic blood pressure was significantly elevated, compared with control animals (116 vs 187 mmHg). The increase in body weight of DOCA-salt rats was smaller than that in control rats. A significant difference between control and DOCA-salt rats with respect to aortic weight was seen at 28 days.

Figure 1a shows the change in aortic IR-ET concentrations in control and DOCA-salt rats. After 7 days of DOCA-salt treatment, a slight increase in aortic IR-ET content was observed compared with age-matched control rats. However, this increase was not statistically significant. After 28 days of DOCA-salt treatment, aortic IR-ET content was significantly higher than in the aged-matched control (2.23 ± 0.37 and 0.93 ± 0.10 ng g^{-1} aortic tissue). On the other hand, no significant changes occurred in renal IR-ET contents of the two experimental groups at 7 and 28 days (control rats; 0.20 ± 0.04 and 0.14 ± 0.01 ng g^{-1} tissue at 7 and 28 days, respectively vs DOCA-salt rats; 0.17 ± 0.02 and 0.13 ± 0.01 ng g^{-1} tissue at 7 and 28 days, respectively, Figure 1b). There was a positive correlation between systolic blood pressure and aortic IR-ET content in the DOCA-salt animals (Figure 2).

The dilution curve of aortic extract clearly revealed a parallel displacement with the standard curve (Figure 3a). To

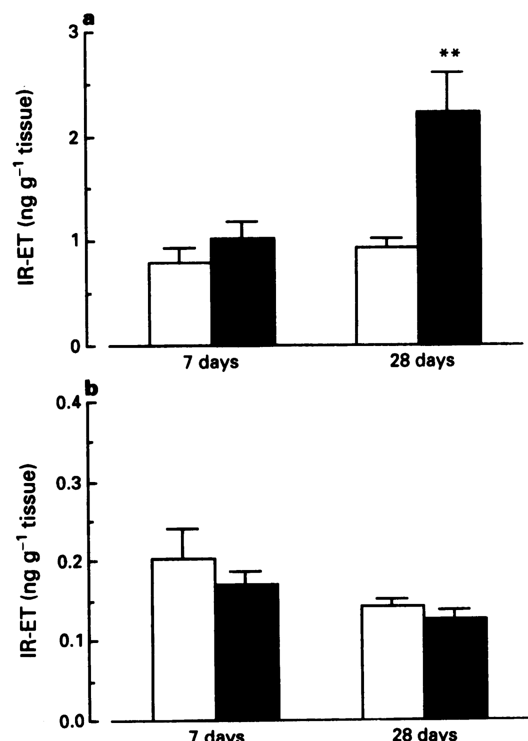

Figure 1 (a) Aortic immunoreactive endothelin (IR-ET) concentrations in rats 7 and 28 days after treatment with deoxycorticosterone (DOCA)-salt (solid columns; $n = 5-11$) and age-matched controls (open columns; $n = 4-14$). (b) Renal IR-ET levels in DOCA-salt ($n = 5-6$) and age-matched controls ($n = 4-6$). Values are mean ± s.e.mean. $^{**}P<0.01$ compared with values of age-matched controls.

Table 1 Effects of deoxycorticosterone acetate (DOCA)-salt treatment of the rat

	Treatment group	n	*SBP* (mmHg)	*Body weight* (g)	*Aorta weight* (mg)
7 days	Control	4	115 ± 5	268 ± 7	40.5 ± 4.9
	DOCA/salt	5	113 ± 6	257 ± 1	41.2 ± 5.2
28 days	Control	14	116 ± 3	361 ± 6	42.4 ± 1.2
	DOCA/salt	11	187 ± 5**	322 ± 11**	52.7 ± 1.9**

n indicates the number of the rats in each group. Values are means ± s.e.mean. **$P<0.01$, compared with values of control rats.

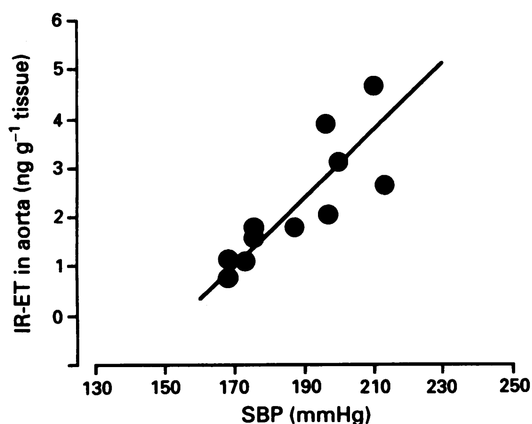

Figure 2 Correlation between concentration of aortic immunoreactive-endothelin (IR-ET) and systolic blood pressure (SBP) in rats 28 days after treatment with deoxycorticosterone acetate (DOCA)-salt. $r = 0.83$; $P<0.01$.

characterize further the IR-ET in aorta of DOCA-salt treated animals, we examined the elution profile of pooled aortic extract on reverse-phase h.p.l.c. coupled with RIA. As shown in Figure 3b, the elution profile revealed one major IR-ET component corresponding to the elution position of synthetic ET-1.

The time course of changes in mean arterial blood pressure (MAP) in control and DOCA-salt treatment after intravenous administration of FR139317 are shown in Figure 4. The average values for MAP after anaesthesia in DOCA-salt rats were significantly higher ($P<0.01$) than in control rats ($n = 9$; 146 ± 6 mmHg vs $n = 12$; 111 ± 4 mmHg). In control rats, MAP was slightly (by about 10–15 mmHg) decreased after intravenous injection of 10 mg kg^{-1} FR139317 compared with vehicle treatment. A significant decrease in MAP was observed 30–60 min after the injection. The MAP of DOCA-salts rats was markedly reduced by FR139317, at the same dose. Significant hypotensive effects were obtained at 15 min and lasted more than 90 min. Maximum responses (about 35 mmHg decrease from basal values) were observed 45–60 min after injection of the antagonist.

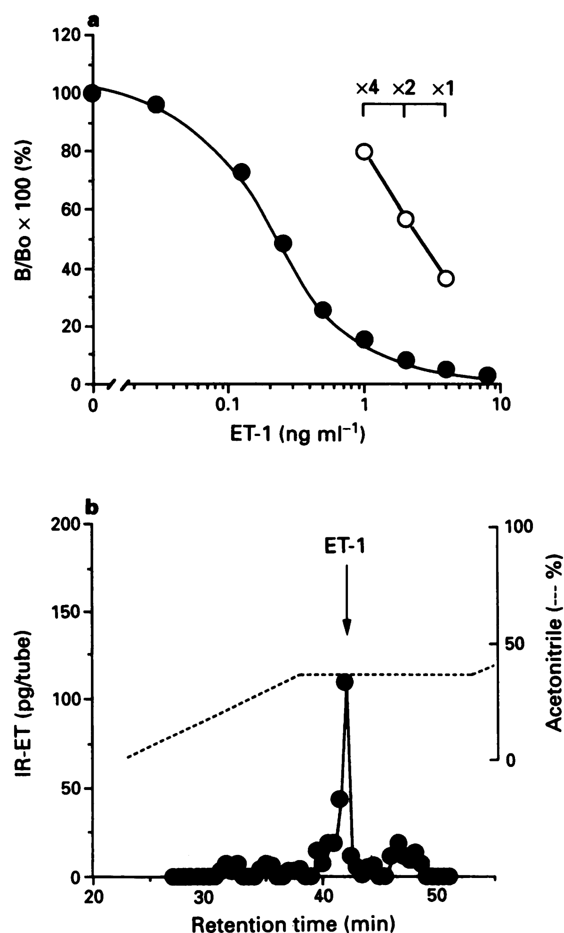

Figure 3 Characterization of immunoreactive-endothelin (IR-ET) in aortic extracts of deoxycorticosterone acetate (DOCA)-salt hypertensive rats. (a) A typical standard curve of endothelin-1 (ET-1) (●) and a dilution curve of the aortic extract (○). (b) Reversed-phase h.p.l.c. profile of IR-ET in aortic extracts from DOCA-salt hypertensive rats. The arrow indicates the elution of synthetic ET-1.

Discussion

The results of the present study clearly demonstrated increased vascular endothelin-1 concentrations in DOCA-salt hypertensive rat. The ET_A receptor antagonist, FR139317, produced a significant decrease in MAP in these DOCA-salt hypertensive rats. As this hypotensive effect was greater than that seen with control rats, the specificity of the finding in DOCA-salt hypertensive rats seem clear. It is most likely that ET-1 makes an important contribution to the maintenance of DOCA-salt-induced hypertension through interaction of the peptide with the ET_A receptor.

We observed an increase aortic ET-1 concentration in association with the hypertension induced by DOCA-salt treatment. Since circulation ET-1 concentrations were not increased in DOCA-salt hypertensive rats, except for the malignant model (Suzuki *et al.*, 1990; Kohno *et al.*, 1991), it seems that the increase in aortic ET-1 is due to a local increased production of the peptide in vascular tissue. We found a significant correlation between the aortic ET-1 level and the systolic blood pressure. These results suggest that ET-1 has a role in the regulation of blood pressure. On the other hand, there were no significant changes in ET-1 levels in the kidney, with DOCA-salt treatment. However, kidney is heterogeneous organ and the homogenates include vascular tissue, tubular epithelial cells and interstitial cells etc., so that it is impossible to estimate the ET-1 content of the renal vasculature from the whole kidney ET-1 level. Therefore, our results do not exclude the possibility that intrarenal vascular ET-1 concentration is altered.

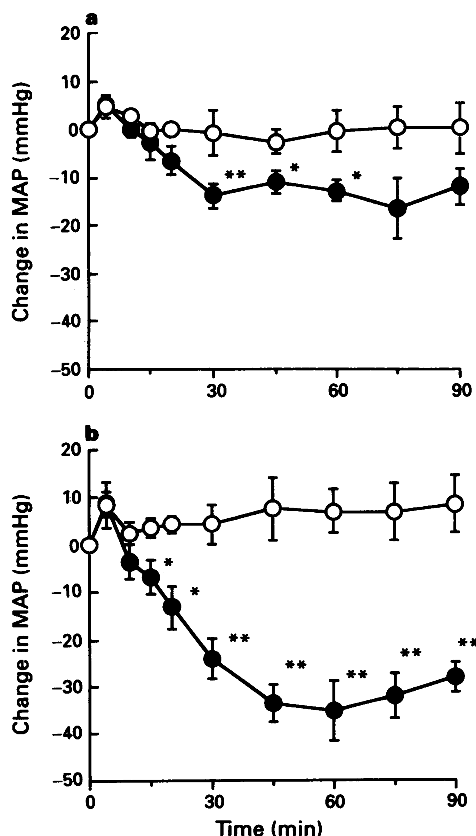

Figure 4 The effects of FR139317 (●; $n = 5-6$) or vehicle (○; $n = 4-6$) on mean arterial pressure (MAP) in anaesthetized (a) control and (b) deoxycorticosterone acetate (DOCA)-salt hypertensive rats. FR139317 was administered as an i.v. bolus injection ($10\ mg\ kg^{-1}$). Each point represents the mean ± s.e.mean. $^*P<0.05$; $^{**}P<0.01$ compared with values of vehicle treatment at the same time.

The question arises as to whether the increase in aortic ET-1 levels in DOCA-salt hypertensive rats is merely the result of hypertension. From our results, the possibility that hypertension itself can induce the vascular ET-1 production cannot be ruled out. However, Larivière *et al.* (1993) noted that vascular ET-1 was not increased in spontaneous hypertensive rats, thereby suggesting that hypertension itself does not enhance ET-1 production in vascular tissues.

There is considerable evidence that many factors, including transforming growth factor-β_1 (TGF-β_1), thrombin, and a vasoactive agent such as angiotensin II and arginine-vasopressin (AVP) can stimulate the expression of prepro ET-1 mRNA in cultured endothelial cells (Kurihara *et al.*, 1989; Imai *et al.*, 1992; Umekawa *et al.*, 1994). It has been reported that AVP plays an important role in DOCA-salt hypertensive rats (Crofton *et al.*, 1979). Mohring *et al.* (1977) observed that plasma concentrations of AVP were elevated in this model of hypertension. Thus, AVP may be one possible candidate for the factor that enhances ET-1 production in the vascular wall. In a study by Sarzani *et al.* (1989), aortic TGF-β_1 mRNA levels were increased in DOCA-salt rats; this growth factor may also be responsible for the increased aortic ET-1 concentrations.

Several ET receptor antagonists have been developed and these will be used to evaluate pathophysiological roles of endogenous ET-1 and may provide a new therapeutic approach for cardiovascular diseases. FR139317 is a selective ET_A receptor antagonist which inhibits ET-1-induced vasoconstrictor effects *in vitro* and *in vivo* (Sagabe *et al.*, 1993). This antagonist ameliorates cerebral vasospasm after subarachnoid haemorrhage (Nirei *et al.*, 1993). It has also been reported that FR139317 can protect against the injury in rats with extensive renal mass reduction, a model of progressive renal disease (Benigni *et al.*, 1993). These results demonstrate the efficacy of FR139317 as an ET_A receptor antagonist for investigating the pathophysiological role of ET-1. In the present study, to clarify further the functional significance of increased vascular ET-1 levels, we evaluated the antihypertensive effect of FR139317 in DOCA-salt hypertensive rats. The result clearly indicated that FR139317 produced a significant hypotensive effect in anaesthetized DOCA-salt hypertensive rats. This hypotensive effect had a slow-onset and was long lasting, a finding consistent with a study which showed that the slow reversal of the vasoconstrictor effects of ET-1 is caused by another ET_A receptor antagonist, BQ123, *in vitro* and *in vivo* (Warner *et al.*, 1994). These authors observed that it takes about 50 min for the antagonist to reverse the established pressor response to ET-1 in anaesthetized rats.

Our results obtained with anaesthetized DOCA-salt hypertensive rats agreed with the finding that another ET_A receptor antagonist, BQ123, produces a small but significant hypotensive effect in conscious DOCA-salt hypertensive rats (Bazil *et al.*, 1992). It has been reported that phosphoramidon, an endothelin converting enzyme inhibitor (Matsumura *et al.*, 1990a), produces a reduction of blood pressure in DOCA-salt hypertensive rats, by inhibiting endothelin bioconversion (Vemulapalli *et al.*, 1993). Stein *et al* (1994) reported that the orally active ET_A receptor antagonist, 5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-naphthalenesulphonamide, produced a significant decrease in blood pressure, in DOCA-salt hypertensive rats. These results show the importance of ET-1 and the ET_A receptor in the maintenance of DOCA-salt induced-hypertension.

In the present study, we observed that FR139317 produced a slight but significant decrease in MAP in control normotensive rats. This would suggest a role of ET-1 and the ET_A receptor in the maintenance of normal blood pressure. However, a previous report indicated that an acute bolus injection of FR139317 had no effect on blood pressure in conscious normotensive rats (Sogabe *et al.*, 1993). BQ123 also had no significant effect of blood pressure in conscious normotensive rats (Bazil *et al.*, 1992; Nishikibe *et al.*, 1993). One explanation for this discrepancy may relate to the experimental condition (anaesthetized rat vs conscious rat). It has been reported that the standard experimental technique such as surgery produces a significant increase in circulating ET-1 (Pollock *et al.*, 1993). Therefore, circulating ET-1 may be increased in anaesthetized rats. Other investigators reported that BQ123 produced a significant decrease in blood pressure in anaesthetized normotensive rats (Bigaud & Pelton 1992; Pollock & Opgenorth, 1993).

Our conclusions regarding the involvement of ET-1 and ET_A receptor in DOCA-salt hypertension depend on the specificity of FR139317 as an ET_A receptor antagonist. In the present study, FR139317 reduced MAP in normotensive rats as well as DOCA-salt hypertensive rats, although the reduction of MAP in the former was small, compared with that in the latter. Since any nonspecific depressor agent would be expected to have a greater hypotensive effect in animals with higher resting blood pressure, one may be doubtful of the specificity of FR139317 for DOCA-salt hypertension. However, we noted that FR139317 treatment to anaesthetized 2K-1C renal hypertensive rats produces only a moderate hypotensive effect to the same degree as that in anaesthetized normotensive rats (unpublished observation). Thus, it seems likely that the greater responses of DOCA-salt hypertensive rats are not due to a nonspecific depressor effect of the agent. In addition, the dose of FR139317 used in our study has been shown to have no effect on the initial depressor response to ET-1, which is mainly mediated by the ET_B receptor (Sogabe *et al.*, 1993). It is reasonable to consider that the antihypertensive effect of FR139317 in DOCA-salt hypertensive rat could be due to an ET_A receptor antagonism.

In conclusion, our results suggest that there is an increased vascular production of ET-1 in DOCA-salt hypertensive rats. In addition, our results indicate the pathophysiological significance of increased endogenous ET-1 in the maintenance of DOCA-salt induced hypertension, through interaction of this peptide with the ET_A receptor.

This study was supported in part by a Grant-in Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan; the Uehara Memorial Foundation; and the Science Research Promotion Fund of Japan Private School Promotion Foundation. We are grateful to Dr Marvin R. Brown, Department of Medicine, University of California, San Diego, U.S.A. for providing antiserum for ET-1, and to M. Ohara for critical comments.

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(Received August 8, 1994
Revised September 30, 1994
Accepted October 27, 1994)