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Role of endothelin-1 in hypertension induced by long-term inhibition of nitric oxide synthase

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Abstract

We examined the effect of long-term nitric oxide (NO) synthase inhibition on vascular and renal endothelin-1 levels and evaluated the antihypertensive effect of endothelin ET_A receptor antagonist FR139317 ((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]-carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl]amino-3-(2-pyridyl) proprionic acid] on rats in which NO synthase was blocked. Chronic NO blockade was produced by oral administration of the NO synthase inhibitor N^G-nitro-L-arginine for 4 weeks, which produced sustained hypertension. At the end of this time, there were no significant changes in aortic and renal immunoreactive-endothelin levels between N^G-nitro-L-arginine-treated hypertensive rats and normotensive control rats. Intravenous injection of FR139317 (10 mg/kg), which had a sufficient hypotensive effect on deoxycorticosterone acetate-salt hypertensive rats, to N^G-nitro-L-arginine-treated hypertensive rats produced only a moderate hypotensive effect, to the same degree as seen in normotensive rats. The results indicate that long-term NO synthase inhibition did not affect vascular and renal endothelin-1 levels in these rats. It seems likely that endothelin-1 and ET_A receptors do not contribute to the sustained hypertension induced by NO synthesis blockade.

Keywords: Endothelin-1; Endothelin ET_A receptor; Nitric oxide (NO); FR139317; Nitro-L-arginine; Hypertension; (Rat)

1. Introduction

Vascular endothelial cells produce vasodilators such as endothelium-derived relaxing factor (EDRF). These cells synthesize and release vasoconstrictor endothelin-1. Endothelin-1 is a 21 amino acid peptide isolated from cultured supernatant of porcine vascular endothelial cells (Yanagisawa et al., 1988). Since endothelin-1 is a most potent endogenous vasoconstrictor substance and has unique pharmacological properties (Rubanyi and Botelho, 1991), endothelin-1 may play a role in the various cardiovascular related disorders such as acute renal failure (Kon and Badr, 1991), cerebral vasospasm (Matsumura et al., 1990a; Nirei et al., 1993) and hypertension (Lüscher et al., 1993; Vanhoutte, 1993).

The mRNA for prepro endothelin-1 is expressed in cultured endothelial cells in vitro and in intact blood vessels in vivo (Yanagisawa et al., 1988). Many factors including transforming growth factor-β, thrombin and vasoactive agents such as angiotensin II and arginine-vasopressin induce the expression of prepro endothelin-1 mRNA in cultured endothelial cells (Imai et al., 1992; Kurihara et al., 1989; Umekawa et al., 1994). In addition, the production of endothelin-1 is regulated by inhibitory stimuli. One potential inhibitory regulator of endothelin-1 production in endothelial cells is nitric oxide (NO). Several in vitro studies indicated that NO reduces the formation of endothelin-1 via a cyclic GMP-dependent mechanism (Boulanger and Lüscher, 1990; Yokokawa et al., 1993). It has also been reported that NO antagonizes vasoconstrictive effects of endothelin-1 (Hirata et al., 1991; Lang and Lewis, 1991).

Although these findings suggest the pathophysiological importance of NO and endothelin-1 interaction in regulation of blood pressure, it is not clear whether long-term NO synthase inhibition increases endothelin-1 production in vivo and induces endothelin-1-dependent hypertension. We examined the effect of long-term NO synthase inhibition on vascular and renal endothelin-1 levels in rats and also effects of blockade of the endothelin-A (ET_A) receptor on blood pressure on rats in which NO synthase was blocked.

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2. Materials and methods

2.1. N^G-Nitro-L-arginine treatment and blood pressure measurement

Experiments were done on male Sprague-Dawley rats, weighing 160–180 g. For NO synthase inhibition, these rats were given drinking water containing N^G-nitro-L-arginine at a concentration of 2.74 mM (Dananberg et al., 1993). Control animals received plain tap water throughout the study. Systolic blood pressure was monitored using a tail cuff and a pneumatic pulse transducer. The rats were exsanguinated 4 weeks after treatment and tissue concentrations of endothelin-1 were determined.

2.2. Tissue extraction and endothelin-1 measurement

Endothelin-1 was extracted from the kidney, according to our method described elsewhere (Fujita et al., 1994). Briefly, kidneys were weighed and homogenized for 60 s in 8 vols. ice-cold organic solution (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 aortas (4 cm) were removed from animals, rapidly cleaned of fat and adherent connective tissue, weighed and homogenized for 60 s in the 4 ml ice-cold organic solution described above. The homogenates were left overnight at 4° C, then 0.4 ml of distilled water was added. Those aortic or renal homogenates were then centrifuged at 3000 rpm for 30 min and the supernatant was stored. Aliquots of the supernatant were diluted 1/10 with a 0.09% trifluoroacetic acid solution and applied to Sep-Pak C18 cartridges. The sample was eluted with 3 ml of 63.3% acetonitrile and 0.1% trifluoroacetic acid. Eluates were dried in a centrifugal concentrator and the dried residue was reconstituted in buffer needed for radioimmunoassay. The clear solution was subjected to radioimmunoassay. Recoveries of endothelin-1 from aorta and renal tissues in our extraction procedures were approximately 80%.

Radioimmunoassay for endothelin-1 was done as described elsewhere (Matsumura et al., 1990b). The limit of detection of endothelin-1 in this assay was 3 pg/tube. Endothelin-1 antiserum (a generous gift from Dr. Marvin R. Brown, Department of Medicine, University of California, San Diego, CA, USA) did not cross-react with big endothelin-1, as described (Hexum et al., 1990).

2.3. Effect of FR139317, an ET_A receptor antagonist

Experiments were done on rats treated with N^G-nitro-L-arginine for 4 weeks and on age-matched control rats. We also examined the effect of ET_A receptor blockade on other two experimental hypertensive models. To prepare 2-kidney 1-clip (2K1C) rats, a silver clip (0.2 mm i.d.) was placed on the left renal artery of male Sprague-Dawley rats weighing about 220 g, following anesthetization with pentobarbital. These 2K1C rats were used 4 weeks after this procedure. Deoxycorticosterone acetate (DOCA)-salt hypertensive rats were prepared as described previously (Fujita et al., 1995). Briefly, male uninephrectomized Sprague-Dawley rats weighing about 220 g were treated twice weekly with DOCA suspended in corn oil, administered subcutaneously (15 mg/kg) and 1% NaCl was added to their tap water for drinking for 4 weeks.

These animals were anesthetized with sodium thiobutabarbital (Inactin, 100 mg/kg 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) or vehicle was administered intravenously by slow bolus injection (2 min). The dose of FR139317 used in this study have been shown to produce complete inhibition of endothelin-1-induced pressor action (Sogabe et al., 1993). Blood pressure was recorded continuously on a polygraph (Nihon Koden, RM 6000G, Tokyo, Japan).

2.4. Drugs

FR139317 ((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]-carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl]amino-3-(2-pyridyl) was a kind gift from Fujisawa Pharmaceutical Co., Osaka, Japan. FR139317 was dissolved in 1 N NaOH and diluted with saline. N^G-Nitro-L-arginine was purchased from Peptide Institute, Osaka, Japan. Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Table 1
Effects of N^G-nitro-L-arginine (NOARG) treatment of rats

Treatment group	*n*	SBP (mm Hg)	Body weight (g)	Aorta weight (mg)	Kidney weight (g)
Control	6	117 ± 4	321 ± 6	40.3 ± 1.0	1.04 ± 0.03
NOARG	5	173 ± 9 [a]	304 ± 8	42.4 ± 1.8	1.02 ± 0.03

n indicates the number of rats in each group. Values are means ± S.E.M. [a] $P < 0.01$, compared with the values of control rats.

2.5. Statistical analysis

All values were expressed as mean ± S.E.M. For statistical analysis, we used the unpaired Student's *t*-test for two-group comparisons and one-way analysis of variance combined with Dunnett's multiple range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

Table 1 summarizes the comparative data on groups of rats treated with N^G-nitro-L-arginine for 4 weeks and of age-matched controls. The administration of N^G-nitro-L-arginine induced a significant increase in systolic blood pressure in the rats, averaging 148% of control values. Two groups displayed an almost identical pattern of weight gain throughout the protocol. There were no significant differences in aortic and kidney weight of control animals and those treated with N^G-nitro-L-arginine for 4 weeks.

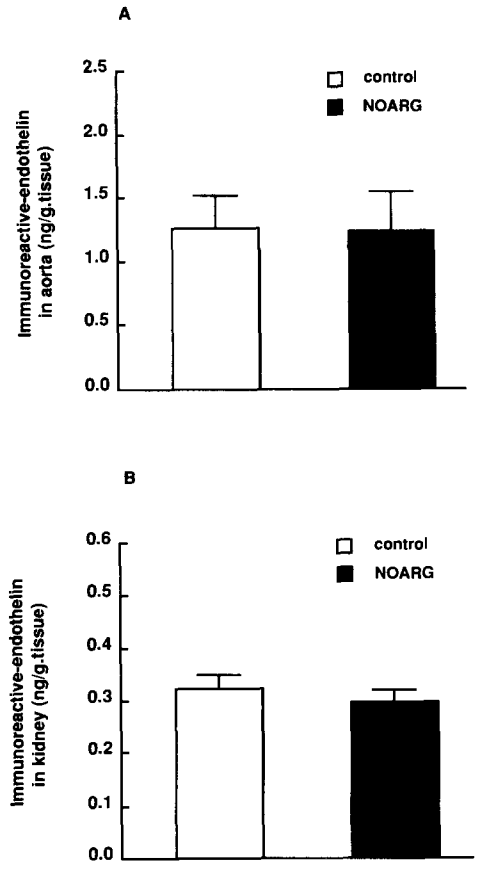

Fig. 1. Bar graphs show (A) aortic and (B) renal immunoreactive endothelin concentrations in rats treated with N^G-nitro-L-arginine (NOARG) for 4 weeks ($n = 5$) and age-matched controls ($n = 6$). Each column and bar represents the mean ± S.E.M.

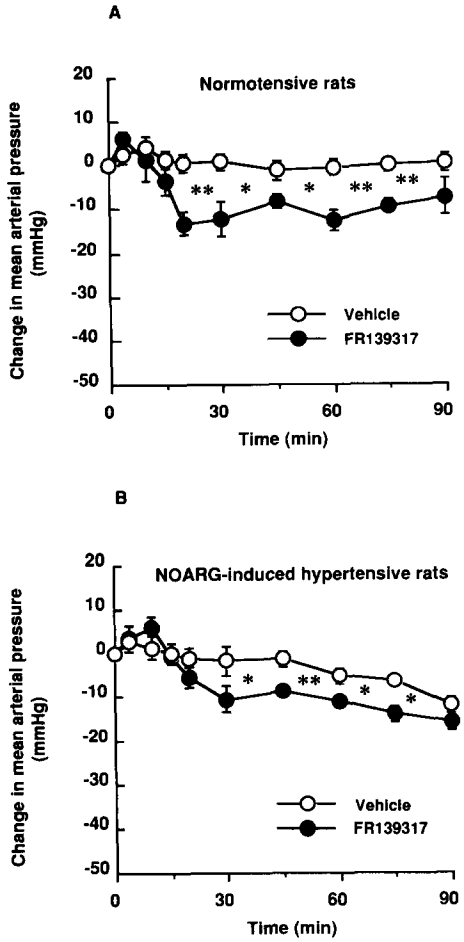

Fig. 2. The effects of FR139317 ($n = 4$–4) or vehicle ($n = 4$–4) on mean arterial pressure in anesthetized (A) control and (B) N^G-nitro-L-arginine (NOARG)-induced hypertensive rats. FR139317 was administered as an i.v. bolus injection (10 mg/kg). Each point represents the mean ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$ compared with values of vehicle treatment at the same time.

Fig. 1A shows the change in aortic immunoreactive-endothelin concentrations in control and N^G-nitro-L-arginine-treated rats. No significant changes occurred in aortic immunoreactive-endothelin contents of the two experimental groups (control rats, 1.26 ± 0.25 ng/g tissue vs. N^G-nitro-L-arginine-treated rats, 1.23 ± 0.32 ng/g tissue, respectively). Similarly, there was no significant difference in renal immunoreactive-endothelin content of the two experimental groups (control rats, 0.32 ± 0.02 ng/g tissue vs. N^G-nitro-L-arginine-treated rats, 0.30 ± 0.02 ng/g tissue, respectively. Fig. 1B).

Time-course changes in mean arterial blood pressure in control and N^G-nitro-L-arginine-treated rats after intravenous administration of FR139317 (10 mg/kg i.v.) are shown in Fig. 2. The average values for mean arterial blood pressure after anesthesia in N^G-nitro-L-arginine-treated rats were significantly higher ($P < 0.01$) than that for control rats ($n = 8$, 167 ± 5 mm Hg vs. $n = 8$, 116 ± 2). In control rats, mean arte-

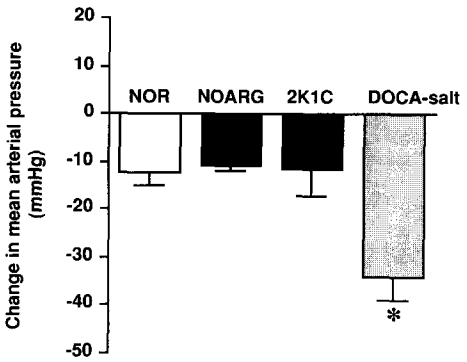

Fig. 3. The hypotensive effects of FR139317 on anesthetized normotensive rats and on three models of experimental hypertensive rats. Normotensive (NOR) rats ($n = 4$), N^G-nitro-L-arginine (NOARG)-induced hypertensive rats ($n = 4$), 2-kidney 1-clip (2K1C) renal hypertensive rats ($n = 4$) and deoxycorticosterone acetate (DOCA)-salt hypertensive rats ($n = 5$). FR139317 was administered as an i.v. bolus injection (10 mg/kg). Results indicate values 60 min after the FR139317 administration. Each column and bar represents the mean ± S.E.M. $^*P < 0.05$ compared with values of NOR rats.

rial blood pressure was slightly (by about 10–15 mm Hg) decreased after intravenous injection of FR139317 compared with vehicle treatment. A significant decrease in mean arterial blood pressure was observed 20–75 min after the injection. The mean arterial blood pressure of N^G-nitro-L-arginine-treated rat was also reduced by FR139317 and the hypotensive effect was to the same degree as that in normotensive rats.

Fig. 3 shows the comparative hypotensive effects of FR139317 (10 mg/kg) on N^G-nitro-L-arginine-treated hypertensive rats and on other two experimental hypertensive rats. The basal mean arterial blood pressure before the administration of the drug was 163 ± 4 mm Hg and 146 ± 6 mm Hg in 2K1C renal hypertensive rats and DOCA-salt hypertensive rats, respectively. In 2K1C rats, the hypotensive effect of FR139317 was to the same degree as that in normotensive rats. In DOCA-salt rats, a potent hypotensive effect was observed after injection of FR139317.

4. Discussion

Long-term inhibition of NO synthase has no apparent effects on vascular and renal endothelin-1 levels in the rat. In addition, intravenous injection of the ET_A receptor antagonist FR139317 to NO synthase-blocked hypertensive rats produced only a moderate hypotensive effect, to the same degree as that in normotensive rats. These results suggest that endothelin-1 and the ET_A receptor do not contribute to the sustained hypertension induced by blockade of NO synthesis.

Our study confirms several previous studies indicating that chronic NO synthase blockade produces sustained systemic hypertension (Dananberg et al., 1993; Riberio et al., 1992). It has been proposed that this hypertension is due to an imbalance between endogenous vasoconstrictors and the diminished vasodilating effect of NO. Several candidates for endogenous vasoconstrictors that may contribute to sustained hypertension induced by NO blockade have been reported (Matsuoka et al., 1994; Pollock et al., 1993; Qiu et al., 1994). However, there is little information for endothelin-1 and NO interaction in this model of hypertension. Several in vitro studies provided evidence that NO is probably an inhibitory modulator of endothelin-1 production in vascular endothelial cells (Boulanger and Lüscher, 1990; Yokokawa et al., 1993). It has been also reported that NO is an inhibitory modulator of endothelin-1-induced vasoconstrictive action (Hirata et al., 1991; Lang and Lewis, 1991). These findings suggest that hypertension with impaired formation of NO may be partly explained by the enhanced endothelin-1 production and/or action occurring under these conditions.

Since the concentration of circulating endothelin-1 is low, and has little cardiovascular action, the peptide has to be regarded as a local factor rather than a circulating hormone. The change in endothelin-1 content in several tissues occurs without change in circulating endothelin-1 levels (Hughes et al., 1992; Larivière et al., 1993). Therefore, tissue endothelin-1 levels rather than circulating endothelin-1 levels are a more accurate indicator for investigating changes in regional endothelin-1 production in vivo. In the present study, long-term NO synthase inhibition had no effect on aortic immunoreactive-endothelin levels in rats. It is unlikely that this lack of effect of NO synthase inhibition is due to an insufficient blockade of NO synthase, since acute intraperitoneal injection of N^G-nitro-L-arginine did not add to the hypertensive effect in animals pretreated with oral N^G-nitro-L-arginine, at the concentration used in this study (Dananberg et al., 1993), thereby suggesting that NO synthase is completely inhibited. We verified the elution profile of one major immunoreactive-endothelin component in aortic extracts corresponding to the elution position of synthetic endothelin-1 by HPLC analysis (Fujita et al., 1995), hence, our result suggests that NO does not have a major role in the regulation of aortic endothelin-1 levels in vivo. However, since we did not examine the effect on other resistance vessels, our results do not exclude the possibility that endothelin-1 levels in other resistance vessels are altered.

Recently, we noted that aortic endothelin-1 content is significantly increased in DOCA-salt hypertensive rats compared with that in age-matched control rats (Fujita et al., 1995). The mechanism for increased vascular endothelin-1 levels in this hypertensive rat is unclear. Studies have demonstrated that endothelium-

dependent relaxation is markedly attenuated in the DOCA-salt hypertensive rat (DeVoorde and Leusen, 1986; Kirchner et al., 1993; Lockette et al., 1986; Hayakawa et al., 1994). Impairment of the relaxation response to EDRF in DOCA-salt hypertension could occur through several mechanisms. This attenuated EDRF response in DOCA-salt hypertensive rats may reflect a decrease in NO production or release (Kirchner et al., 1993; Hayakawa et al., 1994). All these findings suggest that increased vascular endothelin-1 levels in DOCA-salt hypertensive rats may be due to decreased NO production or release in vascular tissues. However, this possibility can probably be ruled out, since inhibition of long-term NO synthase does not increase vascular endothelin-1 levels.

Endothelin-1 has diverse biological activities in the kidney. Administration of endothelin-1 into the renal artery in intact animals induces intense renal vasoconstriction (Matsumura et al., 1989; Miura et al., 1991), and endothelin-1 has a direct effect on renal tubules (Zeidel et al., 1989; Oishi et al., 1991). Endothelin-1 is produced in the kidney by both endothelial and non-endothelial cells, including mesangial (Sakamoto et al., 1990) and tubular epithelial cells (Shichiri et al., 1989; Kohan, 1991). Therefore, we examined the effect of long-term NO synthesis inhibition on levels of renal tissue endothelin-1. The inhibition of long-term NO synthesis had no effect on the renal immunoreactive-endothelin content. We verified by HPLC analysis that this immunoreactive-endothelin component eluted as one major (endothelin-1) and another small (probably the Met^7 sulfoxide form of endothelin-1) peak. Therefore, these results indicate that long-term NO synthesis inhibition has no apparent effect on renal endothelin-1 levels. However, the kidney is a heterogeneous organ with tissue homogenates including vascular tissue, tubular epithelial cells and interstitial cells, etc. Thus it is impossible to estimate endothelin-1 content in renal vasculature based on whole kidney endothelin-1 levels. In addition, endothelin-1 production in vascular endothelial cells and tubular epithelial cells is differently regulated (Kohan and Padilla, 1993). Therefore, we cannot exclude the possibility that there are differential changes in endothelin-1 levels in the several production sites of kidney.

The role of endothelin-1 in blood pressure regulation is controversial (Lüscher et al., 1993; Vanhoutte, 1993). Kurihara et al. (1994) reported that targeted disruption of the mouse endothelin-1 gene, which results in the death of homozygotes, produced elevated blood pressure in heterozygotes. They suggested that endothelin-1 may play a role in the regulation of blood pressure as a depressor rather than as a pressor in mice. On the other hand, several studies using an endothelin receptor antagonist revealed the importance of endothelin-1 in the pathogenesis of some models of experimental hypertension (Nishikibe et al., 1993; Crozel et al., 1993; Stein et al., 1994; Fujita et al., 1995). Therefore, enhanced production and/or action of endothelin-1 in blood vessel may produce hypertension mediated by ET_A or/and vasoconstrictive ET_B receptor under pathophysiological conditions.

FR139317 is a selective ET_A receptor antagonist which inhibits endothelin-1-induced vasoconstrictive effects in vivo and in vitro (Sogabe et al., 1993). In the present study, the intravenous injection of FR139317 produced a small but significant decrease in blood pressure in NO-blocked hypertensive rats. However, the extent of the hypotensive effect of FR139317 is the same in normotensive control rats. It is unlikely that this small hypotensive effect of FR139317 on NO-blocked hypertensive rats is due to an insufficient dose of the drug. Even with treatment of a much higher dose of FR139317 (30 mg/kg), an additional hypotensive effect was not observed in NO-blocked hypertensive rats (data not shown). In addition, the dose of FR139317 (10 mg/kg) used in this study had a sufficient hypotensive effect in DOCA-salt hypertensive rats. The finding that an ET_A receptor antagonist has a hypotensive effect in DOCA-salt rats is consistent with recent reports (Okada et al., 1994; Stein et al., 1994; Fujita et al., 1995). Our result suggests that endothelin-1 and the ET_A receptor may play a role in the maintenance of blood pressure in rats, at least under the condition of anesthesia. However, endothelin-1 and the ET_A receptor do not appear to play a role in the sustained hypertension induced by NO synthesis blockade.

In conclusion, our results indicate that long-term NO synthase inhibition does not affect vascular and renal endothelin-1 levels in the rat. In addition, it seems likely that endothelin-1 and the ET_A receptor do not contribute to the sustained hypertension induced by the NO synthesis blockade.

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