British Journal of Pharmacology (1995) 115, 1377-1381 © 1995 Stockton Press All rights reserved 0007-1188/95 $12.00

Antihypertensive effect of an endothelin receptor antagonist in DOCA-salt spontaneously hypertensive rats

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1 Endothelin-1 gene expression is enhanced in aorta and mesenteric arteries, and possibly other vessels, of deoxycorticosterone acetate (DOCA)-salt hypertensive rats but is normal or reduced in spontaneously hypertensive rats (SHR). Bosentan, a mixed ET_A/ET_B endothelin receptor antagonist, blunts the development of elevated blood pressure of DOCA-salt hypertensive rats but not in SHR. In this study we investigated whether treatment of DOCA-salt SHR with bosentan would result in blunted rise in blood pressure.

2 SHR, aged 13 weeks, were implanted with silastic containing DOCA and offered 1% saline to drink. Systolic blood pressure was measured by the tail-cuff method. Endothelin-1 mRNA abundance in aorta and mesenteric arteries was measured by Northern blot analysis. Content of immunoreactive endothelin in blood vessels was measured by radioimmunoassay.

3 Systolic blood pressure rose less in bosentan-treated DOCA-salt SHR (to 223 ± 2 mmHg) in comparison to the untreated rats (241 ± 1), a small but significant difference ($P < 0.001$). However, blood pressure of bosentan-treated DOCA-salt SHR was still higher than in age-matched SHR. Endothelin-1 mRNA abundance and content of immunoreactive endothelin were increased in the aorta and the mesenteric arterial bed of DOCA-salt SHR, and were unaffected by treatment with bosentan.

4 These data support the hypothesis of a role of endothelin-1 in blood pressure elevation in this hypertensive model with malignant hypertension. They also support the hypothesis that an antihypertensive effect of the mixed ET_A/ET_B endothelin receptor antagonist, bosentan, is found when experimental hypertensive animals exhibit enhanced endothelin-1 gene expression in blood vessels.

Keywords: Blood pressure; endothelin-1 gene expression; conduit arteries; aorta; mesenteric arteries

Introduction

Endothelin-1, a 21-aminoacid peptide, is a potent vasoconstrictor produced by the endothelium and other tissues (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989). We previously reported that the abundance of endothelin-1 mRNA (Larivière *et al.*, 1993a) and the content of immunoreactive endothelin-1 (Larivière *et al.*, 1993b) are increased in the aorta and mesenteric arteries of deoxycorticosterone acetate (DOCA)-salt hypertensive rats. By immunohistochemistry (Larivière *et al.*, 1993b) and by *in situ* hybridization histochemistry (Day *et al.*, 1995), the enhanced production of endothelin in these blood vessels was shown to occur in endothelial cells of aorta and mesenteric arteries of rats with this form of experimental hypertension. The content of endothelin-1 (Larivière *et al.*, 1993b) and the abundance of endothelin-1 mRNA (Schiffrin *et al.*, 1995) are not increased and may even be lower in blood vessels from spontaneously hypertensive rats (SHR) than that found in Wistar-Kyoto control rats (WKY). We recently reported that when DOCA and salt are given to SHR, even in the absence of unilateral nephrectomy, the resulting DOCA-salt SHR develop malignant hypertension and overexpress endothelin-1 in blood vessels (Schiffrin *et al.*, 1995).

When DOCA-salt hypertensive rats are treated chronically with bosentan, a mixed ET_A/ET_B endothelin receptor antagonist, blood pressure rises less in bosentan-treated rats than in untreated rats (Li *et al.*, 1994). In contrast, treatment of SHR with bosentan does not result in a lowering of blood pressure (Li & Schiffrin, 1995). Since both DOCA-salt hypertensive rats and DOCA-salt SHR exhibit enhanced vascular endothelin-1 expression, we proposed the hypothesis that hypertensive rats which overexpressed endothelin-1 in blood vessels would respond to bosentan, and thus chronic administration of bosentan should blunt the development of malignant hypertension in DOCA-salt SHR.

In this study we therefore investigated whether treatment of DOCA-salt SHR with bosentan would result in a blunted rise in blood pressure. We also examined the abundance of transcripts of the endothelin-1 gene and content of immunoreactive endothelin in conduit and small arteries of SHR and DOCA-salt SHR.

Methods

Animal experiments

The study protocol was approved by the Animal Care committee of the Clinical Research Institute of Montreal and was carried out according to the recommendations of the Canadian Council of Animal Care. Rats were housed under conditions of constant humidity (60%) and temperature (22°C) and subjected to 12 h light/dark cycles. Sixteen SHR (Taconic Farms, Germantown, NY, U.S.A.) aged 13 weeks were treated with DOCA by the method of Ormsbee & Ryan (1973). Under pentobarbitone anaesthesia (40 mg kg^{-1}) (Somnotol, MTS Pharmaceuticals, Cambridge, Ontario, Canada), silicone rubber impregnated with DOCA (Sigma Chemical Co., St-Louis, MO, U.S.A.) (200 mg/rat), was implanted subcutaneously. Rats were offered 1% saline to drink. From the day of implantation of DOCA, rats were offered bosentan, 100 mg kg^{-1} per day, mixed with powdered chow. This oral dose has been demonstrated by other investigators and us to block the action of pressor doses of intravenously injected endothelin-1 for more than 24 h, (Clozel *et al.*, 1994; Li & Schiffrin, 1995). Systolic blood pressure was taken weekly by the tail-cuff

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method, after warming and under slight restraint. Blood pressure was recorded on a Grass model 7 polygraph (Grass Medical Instruments, Quincy, MA) fitted with a 7-P8 preamplifier and a model PCPB photoelectric pulse sensor. The average of three pressure readings was recorded. Eight additional SHR were bought aged 13 weeks only for measurement of immunoreactive endothelin content and endothelin-1 mRNA abundance in blood vessels. All rats were killed by decapitation when aged 16 weeks. Blood was collected into tubes containing potassium edetate for measurement of plasma renin activity and immunoreactive endothelin-1, centrifuged, and stored at –70°C until assayed. A segment of thoracic aorta, 1.5 cm in length, and the complete mesenteric vascular bed were removed and dissected free of fat. Tissues were then snap frozen in liquid nitrogen and stored at –70°C until extraction for measurement of immunoreactive endothelin-1 and extraction of total RNA was performed.

Northern blot analysis

Total RNA was extracted from frozen tissues by a guanidine isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). Total RNA samples (20 μg) were denatured in 1 × running buffer (20 mM 3-[N-morpholino]propane-sulphonic acid (MOPS) (pH 7.0), 6 mM sodium acetate, 1 mM EDTA), 6% formaldehyde, and 50% formamide for 15 min at 65°C. RNA samples were run on a 1.0% agarose gel containing 1 × running buffer for 4 to 5 h. The samples were transferred from the gel to a nylon membrane, Hybond-N (Amersham, Arlington, IL, U.S.A.) by capillary action with 3 M NaCl, 0.3 M sodium citrate (20 × SSC). After blotting, the membranes were dried by baking at 80°C for 2 h. The location of the 18S and 28S ribosomal RNA (rRNA) species were revealed by staining with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5). Membranes were prehybridized at 60°C for 2 h (42°C for the ^{32}P-labelled oligonucleotide probe for the 18S rRNA) in 400 mM sodium phosphate buffer (pH 7.2) containing 5% SDS, 1 mM EDTA, 0.1% bovine serum albumin and 50% formamide. Hybridization with the ^{32}P-labelled probe was carried out for 18–20 h at 60°C. The membranes were washed in 12.5 mM NaCl, 0.1% SDS, 3 times at 72°C for 20 min. The membranes were exposed to Reflection films (Dupont, Mississauga, Ontario, Canada) with intensifying screens at –70°C for 6 days (2 to 4 h for the 18S rRNA). The autoradiograms were analyzed with a Bio-Rad imaging densitometer and the Molecular analyst software version 1.1 (Bio-Rad Laboratories, Hercule, CA, U.S.A.).

The rat endothelin-1 probe was prepared from rat lung RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) (Larivière *et al.*, 1993a). A 319 bp rat preproET-1 PCR product was obtained using a 5′ forward primer: 5′-CTAGGTCTAAGCGATCCTTG-3′, and a 3′ reverse primer: 5′-TTCTGGTCTCTGTAGAGTTC-3′, located at nucleotides 266 to 285 and 565 to 584 of the coding sequence of the rat endothelin-1 cDNA respectively (Sakurai *et al.*, 1991). This PCR product was then cloned into pGEM–7zf(+) plasmid (Promega, Madison, WI, U.S.A.). The radiolabelled antisense riboprobe was prepared as previously described (Larivière *et al.*, 1993a) using [α^{32}P]-UTP (800Ci mmol^{-1}; Dupont). 18S rRNA was analyzed with a specific oligonucleotide probe (5′-CTTCCTCTAGATAGTCAAGTTCGACCGTCT-3) (Torczynski *et al.*, 1983) labelled with T4 polynucleotide kinase (Pharmacia, Baie d'Urfé, Quebec, Canada) and [γ^{32}P]-ATP (3000 Ci mmol^{-1}, Dupont). The ^{32}P-labelled probes were purified by chromatography on a Sephadex G-50 column (Pharmacia) or NACS cartridges (Gibco-BRL, Burlington, Ontario, Canada) for the riboprobe and the oligonucleotide probe respectively.

Measurement of ir-endothelin-1 in plasma and tissues

Plasma endothelin-1 was measured by radioimmunoassay after extraction by passage through a C18 Sep-Pak cartridge, as previously described (Schiffrin & Thibault, 1991). Tissue endothelin-1 immunoreactivity was measured in thoracic aorta and the mesenteric arterial bed as described previously (Larivière *et al.*, 1993b). Plasma renin activity was measured by radioimmunoassay of angiotensin I generated during incubation of plasma at 37°C for 2 h as previously described (Larivière *et al.*, 1993b).

Analysis of data

Results are expressed as means ± s.e.mean. Comparison of mean values was performed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Differences were considered significant when the P value was < 0.05.

Results

Effect of bosentan on systolic blood pressure and plasma endothelin-1

Figure 1 depicts the evolution of systolic blood pressure under treatment with bosentan. Blood pressure rose to malignant levels in DOCA-salt SHR, but rose significantly less in the rats treated with the endothelin receptor antagonist. However, bosentan-treated DOCA-salt SHR presented elevation of blood pressure above that of age-matched SHR not receiving DOCA and salt. Treatment with bosentan did not affect the weight of DOCA-salt SHR. Left ventricular hypertrophy, as shown by the heart to body weight ratio, was similar in bosentan-treated and untreated rats (Table 1). There were no differences in plasma concenrations of immunoreactive endothelin-1 or plasma renin activity between both groups of rats. Results in SHR are provided in Table 1 which show that blood pressure values in DOCA-salt SHR treated with bosentan are different from those of age-matched SHR which had not received DOCA and salt.

Vascular endothelin-1 mRNA and immunoreactivity in DOCA-salt SHR versus SHR

Figure 2 shows a Northern blot of RNA extracted from the aorta and the mesenteric arteries of SHR (systolic blood pressure 195 ± 2 mmHg), and Figure 3 shows results of densitometric analysis of 3–4 experiments from different animals. The 2.3 kb band corresponding to endothelin-1 mRNA exhibited greater intensity in lanes from vessels of DOCA-salt SHR than in those from SHR. Similarly, the content of im-

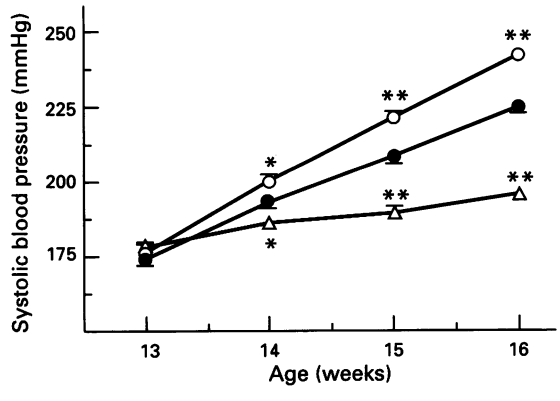

Figure 1 Systolic blood pressure of 8 DOCA-salt treated spontaneously hypertensive rats (SHR) which received bosentan (●), 8 DOCA-salt SHR (○) and 8 untreated SHR which did not receive bosentan (△). *$P < 0.05$; **$P < 0.01$ vs rats treated with bosentan; s.e.mean hidden in part by symbols.

Table 1 Systolic blood pressure, body and heart weight, plasma endothelin-1 and plasma renin activity

Parameter	*DOCA-salt SHR*	*DOCA-salt SHR + bosentan*	*SHR*
Number	7	8	10
Systolic blood pressure (mmHg)	241 ± 1	223 ± 2*	200 ± 2
Body weight (g)	293 ± 3	290 ± 2	350 ± 4*
Heart weight (g)	1.72 ± 0.06	1.67 ± 0.04	1.34 ± 0.02
Heart weight to body weight ratio (%)	0.586 ± 0.017	0.577 ± 0.013	0.38 ± 0.004
Plasma endothelin-1 ($pmol^{-1}$)	2.74 ± 0.25	2.77 ± 0.43	2.51 ± 0.36
Plasma renin activity (ng angiotensin I ml^{-1} min^{-1})	2.12 ± 0.34	2.16 ± 0.59	1.85 ± 0.24

*$P < 0.01$ vs. other groups.

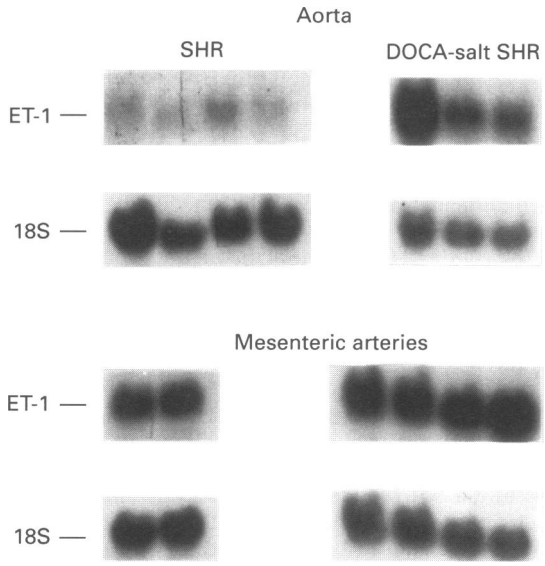

Figure 2 Representative Northern blots of total RNA (20 μg per lane) extracted from the aorta and the complete mesenteric arterial bed from spontaneously hypertensive rats (SHR) and SHR treated with DOCA and salt (DOCA-salt SHR). The upper panel shows a single band of 2.3 kb corresponding to the rat endothelin-1 mRNA transcript. The analysis was done using a specific ^{32}P-labelled complementary RNA probe for rat preproendothelin-1. The lower panel shows the band of the 18S ribosomal RNA on the same blots, obtained by hybridizing with a specific ^{32}P-labelled oligonucleotide probe, and used to normalize the abundance of endothelin-1 mRNA (see Figure 3). Similar results were obtained in at least two different membranes from independent groups of rats.

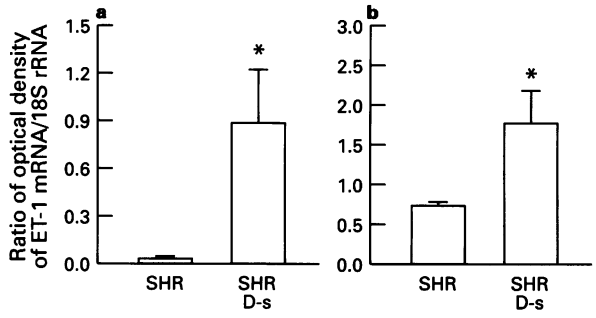

Figure 3 Endothelin-1 mRNA content expressed as the ratio of the optical density of the endothelin-1 mRNA to the 18S ribosomal RNA (rRNA) bands (mean ± s.e.mean) from samples of RNA extracted from the aorta (a) and the mesenteric arterial bed (b) of 6 spontaneously hypertensive rats (SHR) and 6 SHR treated with DOCA and salt (SHR D-s) examined by Northern blot analysis as in Figure 2. *$P < 0.05$. Similar results were obtained in at least two different membranes from independent groups of rats.

munoreactive endothelin-1 was increased in the thoracic aorta and the mesenteric arterial bed of DOCA-salt SHR (Table 2). The wet weight of vessels was greater in the DOCA-salt SHR, as expression of hypertrophy of the vascular wall. Since endothelin overexpression is purely endothelial (Day *et al.*, 1995), the content was not expressed per unit weight but rather per unit length of aorta or in the whole mesenteric arterial bed.

Effect of bosentan on vascular endothelin-1 mRNA abundance

Endothelin-1 mRNA content was measured in the DOCA-salt SHR with or without bosentan treatment. Figure 4 shows that abundance of endothelin-1 mRNA was unchanged in both thoracic aorta segments and the mesenteric arterial bed under treatment with bosentan in DOCA-salt SHR.

Discussion

In previous studies we demonstrated that enhanced expression of the endothelin-1 gene could be detected in different vascular beds in DOCA-salt hypertensive rats (Larivière *et al.*, 1993a,b; Day *et al.*, 1995; Schiffrin *et al.*, 1995) and that SHR did not overexpress endothelin-1 (Schiffrin *et al.*, 1995). We have already shown that when SHR are treated with DOCA and salt and acquire malignant elevation of blood pressure, they exhibit increased vascular abundance of endothelin-1 mRNA (Schif-

Table 2 Wet weight of blood vessels and immunoreactive endothelin-1 (ir-ET-1) content

	Tissue wet weight (mg)		*ir-ET-1 content* (fmol)	
Group	*Aorta*	*Mesenteric arterial bed*	*Aorta*	*Mesenteric arterial bed*
SHR	47 ± 1.7	56 ± 4.0	2.61 ± 0.18	16.2 ± 1.11
DOCA-salt SHR	55 ± 2.2*	127 ± 5.1*	15.5 ± 1.17**	29.7 ± 5.45*

Ir-ET-1 content is expressed as fmol per 1.5 cm length segment of aorta or whole mesenteric arterial bed. Values are means ± s.e.mean ($n = 5-6$ rats per group); ir-ET-1, immunoreactive endothelin-1. *$P < 0.05$, **$P < 0.01$.

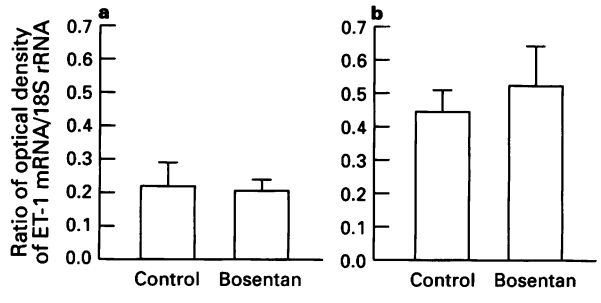

Figure 4 Endothelin-1 mRNA content expressed as the ratio of the optical density of the endothelin-1 mRNA to the 18S ribosomal RNA (rRNA) bands (mean ± s.e.mean) from samples of RNA extracted from the aorta (a) and the mesenteric arterial bed (b) of 6 DOCA-salt spontaneously hypertensive rats (DOCA-salt SHR) treated with bosentan and 7 which did not receive bosentan, examined by Northern blot analysis as in Figure 2.

frin *et al.*, 1995), similar to DOCA-salt hypertensive rats (Larivière *et al.*, 1993a). The present results using the mixed ET_A/ET_B endothelin receptor antagonist, bosentan, which demonstrate blunting, albeit small, of the elevation of blood pressure when DOCA-salt SHR are treated with this agent, suggests that experimental animals exhibit a hypotensive response to endothelin antagonists when they overexpress vascular endothelin-1. This response is independent of plasma concentrations of endothelin, since the levels of the peptide in the circulation are not elevated in these hypertensive models which overexpress the endothelin-1 gene in blood vessels and respond to endothelin antagonism with blood pressure lowering. Since in DOCA-salt SHR blood pressure is still higher than that of age-matched SHR, this suggests that endothelin overexpression is not the only mechanism responsible for the elevation of blood pressure to malignant levels in these rats.

The mechanisms whereby DOCA together with salt induce overexpression of the endothelin-1 gene in unilaterally nephrectomized Sprague-Dawley DOCA-salt hypertensive rats (Day *et al.*, 1995; Schiffrin *et al.*, 1995) and in DOCA-salt-SHR (Schiffrin *et al.*, 1995; this study) are not well understood. It takes 3–4 weeks for the enhancement of expression to become evident in Northern blot analysis of RNA from arteries from DOCA-salt hypertensive rats (Larivière *et al.*, 1994). DOCA-salt rats which have not undergone unilateral nephrectomy and which are slightly less hypertensive, do no exhibit endothelin-1 overexpression in blood vessels. Elevated blood pressure *per se* does not appear to be a factor in the increases in expression of the endothelin-1 gene reported, but could play a permissive role, particularly when elevation is very severe, as in DOCA-salt SHR. Unilateral nephrectomy, which in Sprague-Dawley rats is necessary for the blood pressure to reach very high levels under treatment with DOCA and salt, is not required for SHR receiving DOCA and salt to develop enhanced expression of endothelin-1 in blood vessels. Which of the different factors potentially involved, such as growth factors like TGF-β1 (Kurihara *et al.*, 1989), whose expression is increased in DOCA-salt hypertension (Sarzani *et al.*, 1989), vasopressin (Imai *et al.*, 1992), haemodynamic factors (Yoshizumi *et al.*, 1989), or other factors participate pathogenically in the increases in endothelin-1 expression under the combined influence of DOCA and salt, remains to be established. In DOCA-salt SHR, renal lesions with accumulation of fibrinoid material have been described, which are suggestive of activation of blood coagulation (Sesoko *et al.*, 1984). Thus, thrombin, which is known to stimulate endothelin expression (Yanagisawa *et al.*, 1988), could also play a role in the activation of endothelin-1 expression.

It remains to be established which are the mechanisms through which endothelin antagonism is able to blunt the rise in blood pressure which occurs when SHR are treated with DOCA and salt. From these studies it cannot yet be concluded whether this is the result of an antivasoconstrictor effect of bosentan blocking the vascular actions of endothelins, whether development of vascular hypertrophy (which is severe in these rats, Schiffrin *et al.*, 1995), is curtailed as we showed earlier in DOCA-salt hypertensive rats (Li *et al.*, 1994), whether it results from an antagonism of the renal effects of endothelins (Stacy *et al.*, 1990), or other as yet undetermined mechanisms. Bosentan treatment did not affect endothelin-1 expression in blood vessels, as demonstrated in Figure 4, in DOCA-salt SHR in this study in which only endothelin-1 mRNA abundance was measured. In DOCA-salt hypertensive rats treated with bosentan, we previously showed that immunoreactive endothelin varied proportionally to endothelin-1 mRNA measured by Northern blot analysis, as in the present study (Larivière *et al.*, 1995). Thus, changes in endothelin-1 expression are not involved in the response of DOCA-salt SHR to endothelin antagonist treatment. We (Li & Schiffrin, 1995) and others (Clozel *et al.*, 1994) have shown that bosentan does not antagonize the actions of other vasoconstrictor peptides. In this study we also show that plasma renin activity is unaffected by bosentan treatment. Thus, neither activation nor inhibition of the renin-angiotensin system appear to contribute to the effects of bosentan.

Löffler *et al.* (1993) have previously reported that endothelin plasma levels increase after treatment with bosentan in normal rats. A similar phenomenon was also found in DOCA-salt hypertensive rats (Li *et al.*, 1994). In contrast, administration of this agent did not produce a rise in plasma endothelin immunoreactivity in DOCA-salt SHR in the present study. The reasons for this remain to be established. It is of interest that adult SHR treated with bosentan also do not exhibit a rise in plasma immunoreactive endothelin levels (Li & Schiffrin, 1995), which may indicate an inability for this strain of rats to respond to bosentan treatment with increased plasma concentrations of endothelin.

In conclusion, development of malignant hypertension in SHR which occurs after treatment with DOCA and salt, is attenuated but not completely eliminated by chronic oral administration of the mixed ET_A/ET_B endothelin receptor antagonist bosentan. The sensitivity of this hypertensive model to treatment with an endothelin receptor antagonist is found together with enhanced expression of endothelin-1 in blood vessels. Since Sprague-Dawley DOCA-salt hypertensive rats, which also overexpress vascular endothelin-1, respond with blood pressure lowering to bosentan, whereas SHR, which do not overexpress endothelin-1, do not respond to bosentan, this suggests that a hypotensive response to endothelin antagonism occurs when there is vascular activation of the expression of the endothelin-1 gene.

The authors thank Ms Angie Poliseno for secretarial help. This work was supported by a group grant from the Medical Research Council of Canada (M.R.C.) to the Multidisciplinary Research Group on Hypertension and by grants from the Fondation des maladies du coeur du Québec. Bosentan was a kind gift of Dr Martine Clozet, Pharma Research, F. Hoffmann-LaRoche Ltd., Basel, Switzerland.

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(Received January 26, 1995
Revised April 21, 1995
Accepted May 5, 1995)