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Cardiovascular responses mediated by two types of endothelin ET_B receptor in spontaneously hypertensive and Wistar-Kyoto rats

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Abstract

This study shows the effects of a selective endothelin ET_B receptor agonist, IRL 1720 {Ac-[$Ala^{11,15}$]endothelin-1-(8–21)}, on cardiovascular responses in anesthetized spontaneously hypertensive rats and Wistar-Kyoto rats. Single intravenous bolus injection of IRL 1720 caused a dose-related short-lasting fall in blood pressure, left ventricular pressure and myocardial contractility. However, repeated intravenous bolus injection of 10^{-5} mol/kg IRL 1720 produced a biphasic response consisting of an initial short-lasting decrease followed by a sustained increase in these parameters. The initial decrease was reduced, whereas the following increase was enhanced with the repeated injections of IRL 1720. The cardiovascular pressor response was not inhibited by the endothelin ET_A receptor antagonist, FR139317 ((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl]amino-3-(2-pyridyl)propionic acid). The effects of IRL 1720 were qualitatively similar but more potent in spontaneously hypertensive rats than in Wistar-Kyoto rats. These results suggest the existence of two types of endothelin ET_B receptor for IRL 1720: a tachyphylactic endothelin ET_B receptor that mediates cardiovascular depressor responses and a less tachyphylactic endothelin ET_B receptor that mediates pressor responses in the rat.

Keywords: IRL 1720; Sarafotoxin S6c; Endothelin ET_B receptor; FR139317; Hemodynamics

1. Introduction

Endothelin (Yanagisawa et al., 1988), a modulator of cardiovascular functions, appears to elicit its physiological and pathophysiological effects through at least two types of endothelin receptors. The endothelin ET_A receptor (Arai et al., 1990) is selectively activated by endothelin-1 and is a predominantly smooth muscle cell receptor that induces vasoconstriction, whereas the endothelin ET_B receptor (Sakurai et al., 1990) is unselectively activated by both endothelin-1 and endothelin-3 and is thought to be endothelium associated, mediating vasorelaxation (Rubanyi and Polokoff, 1994). However, recent studies have suggested that there are subtypes of the endothelin ET_B receptor that mediate vasoconstriction (Clozel et al., 1992; Sumner et al., 1992; Moreland et al., 1992; Sudjarwo et al., 1993, 1994).

It has been shown that single intravenous bolus injection of endothelin-1 or endothelin-3 produces an initial decrease followed by an increase in blood pressure (Yanagisawa et al., 1988; Inoue et al., 1989) and that the initial depressor effect is inhibited by a selective endothelin ET_B receptor antagonist, RES-701-1 (cyclic [Gly^1-Asp^9][Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp]) (Tanaka et al., 1994). In addition, Le Monnier de Gouville et al. (1990) reported that the systemic depressor response to endothelin-1 or endothelin-3 is subject to cross-tachyphylaxis in vivo. Once the tachyphylaxis has occurred to the vasodilator activity of endothelin, intravenous bolus injection of endothelin-3 induces only a

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Table 1
Baseline values of cardiovascular functions in anesthetized Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

	BW (g)	HR (bpm)	BP (mm Hg)	LVP (mm Hg)	LV$dp/dt_{max}$ (mm Hg/s)
WKY ($n = 31$)	345.2 ± 6.3	287.5 ± 9.7	59.9 ± 1.9	111.7 ± 1.8	5718.4 ± 296.2
SHR ($n = 29$)	302.1 ± 4.4 [b]	343.8 ± 7.0 [b]	79.1 ± 2.4 [a]	123.5 ± 2.5	6647.1 ± 290.8

BW: body weight, BP: mean arterial blood pressure, LVP: left intraventriculer pressure, LV$dp/dt_{max}$: maximum differential value of left intraventriculer pressure. Values are means ± S.E.M. [a] $P < 0.05$, [b] $P < 0.01$ compared with corresponding values of WKY group.

vasopressor effect. Moreover, the selective endothelin ET_A receptor antagonist, BQ123(cyclo[Asp-Pro-Val-Leu-Trp]), only partially inhibited the pressor effect of endothelin-1 (Ihara et al., 1992; McMurdo et al., 1993).

Although the receptor subtypes responsible for the pressor response to endothelin are poorly understood, it is possible that the endothelin ET_B receptor participates in this response. To test this hypothesis, we studied the in vivo effect of IRL 1720 {Ac-[$Ala^{11,15}$]endothelin-1-(8–21)}, a novel and selective endothelin ET_B receptor agonist (James et al., 1993), on cardiovascular responses in rats. Furthermore, recent studies have documented that the responses of endothelial cells and smooth muscle cells mediated by the endothelin ET_B receptor are greater in spontaneously hypertensive rats than in the Wistar-Kyoto rats (Batra et al., 1993; Yokokawa et al., 1994). Therefore, the effects of IRL 1720 on cardiovascular responses in spontaneously hypertensive rats were compared with those in the Wistar-Kyoto rats.

2. Materials and methods

Male Wistar-Kyoto rats and spontaneously hypertensive rats were obtained from Charles River Japan (Kanagawa, Japan). These rats were anesthetized with urethane (1 g/kg i.p.). The left femoral artery and vein were cannulated with polyethylene catheters for blood pressure measurement and drug injections, respectively. A polyethylene catheter was also inserted into the left ventricle via the left carotid artery for recording left intraventricular pressure. Left intraventricular dp/dt_{max}, as an index of myocardial contractility, was obtained by feeding left intraventricular pressure into a differential amplifier (NEC Sanei, 1309). The electrocardiogram for limb lead II was recorded with a biophysiograph (NEC Sanei, 1206). These data were recorded with an ECG processor (Softron, Tokyo, Japan) and analyzed using an analytical program (Softron SBP-4, Tokyo, Japan) on a personal computer.

A dose-response curve for IRL 1720 (10^{-8}–10^{-5} mol/kg) was obtained by intravenous bolus injections, each successive dose being given at 30–60-min intervals when the changes in the parameters due to the previous injection had returned to their initial values.

Six consecutive intravenous bolus injections of IRL 1720 (10^{-6} or 10^{-5} mol/kg) or 10^{-9} mol/kg sarafotoxin S6c were administered by maintaining a 10-min interval between two doses. This time interval was chosen according to an earlier study to induce tachy-

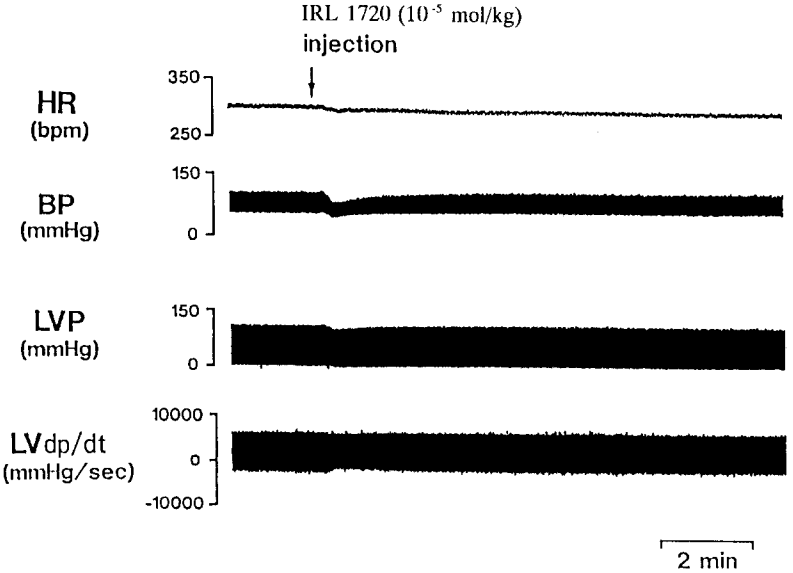

Fig. 1. Representative tracing of effects of IRL 1720 (10^{-5} mol/kg) on heart rate (HR; beat/min), blood pressure (BP; mm Hg), left intraventricular pressure (LVP; mm Hg) and differential value of left intraventricular pressure (LV$dp/dt$; mm Hg/s) in anesthetized Wistar-Kyoto rats.

phylaxis (Le Monnier de Gouville et al., 1990). In a group of rats, the cardiovascular response to an intravenous bolus of acetylcholine (0.5 μg/kg) was tested after the last IRL 1720 injection. In a separate group of rats, after the six intravenous bolus injections of IRL 1720 (10^{-5} mol/kg), an intravenous bolus of FR139317 ((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]ammino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indoyl)]propionyl]amino-3-(2-pyridyl)propionic acid) (10 mg/kg) (Sogabe et al., 1993) was injected to block the ET_A receptor, and 5 min later, an intravenous bolus of IRL 1720 (10^{-5} mol/kg) was administered.

After five intravenous bolus injections of 10^{-5} mol/kg IRL 1720 or 10^{-9} mol/kg sarafotoxin S6c, 10^{-6} mol/kg, 10^{-5} mol/kg IRL 1720, or 10^{-9} mol/kg sarafotoxin S6c was injected to examine the cross-tachyphylaxis of the endothelin ET_B receptors.

IRL 1720 was a gift from Dr. T. Okada, Ciba-Geigy (Japan), Takarazuka, Japan. Sarafotoxin S6c was purchased from the Peptide Institute (Osaka, Japan).

Results were expressed as the means ± S.E.M. Statistical comparisons were made using Student's *t*-test.

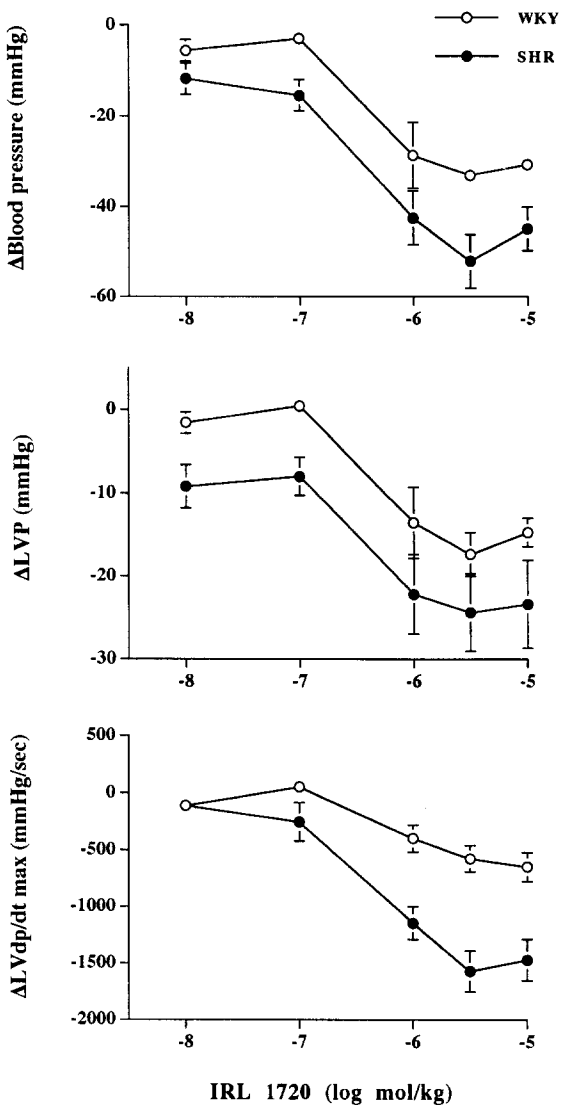

Fig. 2. Dose-response relations for IRL 1720-induced maximum changes of cardiovascular functions from baseline values in anesthetized Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Values represent means ± S.E.M. of eight experiments.

3. Results

3.1. Effects of IRL 1720 on cardiovascular functions

The baseline values of cardiovascular functions in the anesthetized spontaneously hypertensive rats and Wistar-Kyoto rats are summarized in Table 1. Both heart rate and blood pressure in spontaneously hypertensive rats were significantly higher than those in Wistar-Kyoto rats.

As shown in Fig. 1, intravenous bolus injection of IRL 1720 (10^{-5} mol/kg) caused a short-lasting fall in blood pressure, left intraventricular pressure, and left intraventricular dp/dt. The hypotension attained a maximum in 30 s after the injection and disappeared entirely within 3 min. Heart rate was not significantly modified by IRL 1720. Dose-related maximum decreases in blood pressure, left intraventricular pressure, and left intraventricular dp/dt_{max} were observed in both spontaneously hypertensive rats and Wistar-Kyoto rats (Fig. 2). Higher doses of IRL 1720 ($> 10^{-6}$ mol/kg) resulted in significant decreases from control values of these parameters in both spontaneously hypertensive rats and Wistar-Kyoto rats ($P < 0.05$). The effects of IRL 1720 on spontaneously hypertensive rats were qualitatively similar to those in Wistar-Kyoto rats. Although higher doses of IRL 1720 ($> 10^{-6}$ mol/kg) induced larger changes in blood pressure and left intraventricular dp/dt_{max} in spontaneously hypertensive rats than in Wistar-Kyoto rats, these changes were not significant ($P > 0.05$).

3.2. Effects of repeated injections of IRL 1720

The effects of repeated intravenous bolus injections of 10^{-6} mol/kg IRL 1720 on blood pressure, left intraventricular pressure, and left intraventricular dp/dt_{max} in anesthetized spontaneously hypertensive rats and Wistar-Kyoto rats are summarized in Fig. 3. Each injection of 10^{-6} mol/kg IRL 1720 decreased blood pressure, left intraventricular pressure, and left intraventricular dp/dt_{max}. Responses to the first injection of 10^{-6} mol/kg IRL 1720 were weaker than those to the subsequent injections in both Wistar-Kyoto rats (Fig. 3A) and spontaneously hypertensive rats (Fig. 3B).

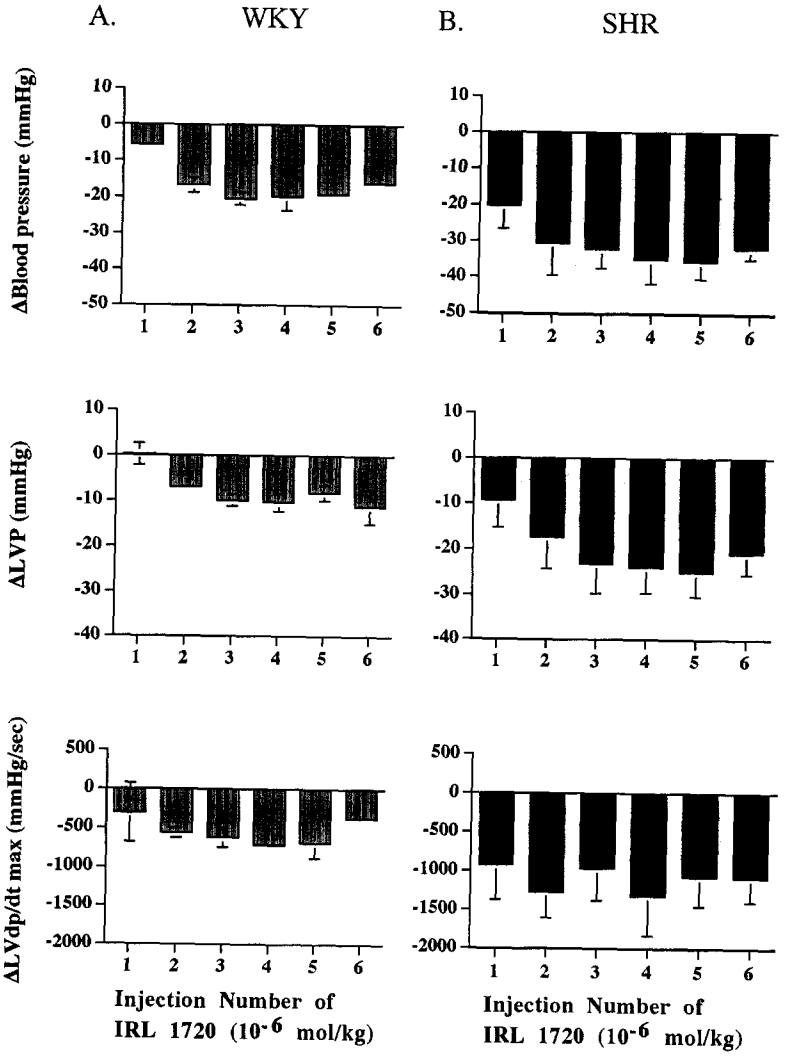

Fig. 3. Effects of six repeated intravenous bolus injections of IRL 1720 (10^{-6} mol/kg) on changes of blood pressure (Δblood pressure), left intraventricular pressure (ΔLVP), and left intraventricular dp/dt_{max} (ΔLVdp/dt max) in anesthetized Wistar-Kyoto rats (WKY; A) and spontaneously hypertensive rats (SHR; B). 10 min elapsed between two doses. Values are means ± S.E.M. of 10 experiments.

The effects of repeated injection of 10^{-5} mol/kg IRL 1720 are shown in Fig. 4. The first and second injections of 10^{-5} mol/kg IRL 1720 induced a monophasic decrease in blood pressure, left intraventricular pressure, and left intraventricular dp/dt_{max} in both Wistar-Kyoto rats (Fig. 4A) and spontaneously hypertensive rats (Fig. 4B). The third injection, however, produced a biphasic response consisting of an initial short-lasting decrease followed by a sustained increase in these parameters in both strains of rats.

Table 2
Effects of acetylcholine on cardiovascular functions before and after repeated injections of 10^{-5} mol/kg IRL1720 in anesthetized Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

	ΔBP (mm Hg)		ΔLVP (mm Hg)		$\Delta LVdp/dt_{max}$ (mm Hg/s)	
	Before	After	Before	After	Before	After
WKY	−20.1 ± 5.3	−16.7 ± 3.4	−8.5 ± 1.8	−7.1 ± 0.3	−957.6 ± 289.7	−1116.8 ± 324.2
SHR	−23.2 ± 7.8	−21.1 ± 5.8	−15.3 ± 5.1	−11.3 ± 2.2	−883.4 ± 197.3	−721.1 ± 146.7

Acetylcholine (0.5 μM/kg) was applied before and after six repeated injections of IRL 1720 (10^{-5} mol/kg). ΔBP: changes of mean arterial pressure; ΔLVP: changes of left intraventricular pressure; $\Delta LVdp/dt_{max}$: changes of maximum differential value of left intraventricular pressure. Values are means ± S.E.M. of five experiments.

The initial decrease was reduced, whereas the following increase was enhanced with the repeated injections.

Intravenous bolus injection of acetylcholine decreased blood pressure, left intraventricular pressure, and left intraventricular dp/dt_{max} (Table 2). The effects of acetylcholine (0.5 μg/kg) were not modified after six intravenous bolus injections of IRL 1720.

3.3. Effects of the endothelin ET_A receptor antagonist

After six intravenous bolus injections of 10^{-5} mol/kg IRL 1720, an endothelin ET_A receptor antagonist, FR139317 (10 mg/kg i.v.), was injected to identify the endothelin receptor subtype mediating the increased cardiovascular responses. In the rats treated with FR139317, changes in parameters of cardiovascular functions were similar to those induced by the sixth injection of IRL 1720 (data not shown, $n = 5$, $P > 0.05$).

3.4. Effects of repeated injections of sarafotoxin S6c

The effects of repeated intravenous bolus injections of sarafotoxin S6c (10^{-9} mol/kg) on blood pressure, left intraventricular pressure, and left intraventricular dp/dt_{max} in anesthetized spontaneously hypertensive rats and Wistar-Kyoto rats are shown in Fig. 5. The first injection of sarafotoxin S6c induced a biphasic response consisting of a transient decrease followed by a long-lasting increase in these parameters. In contrast,

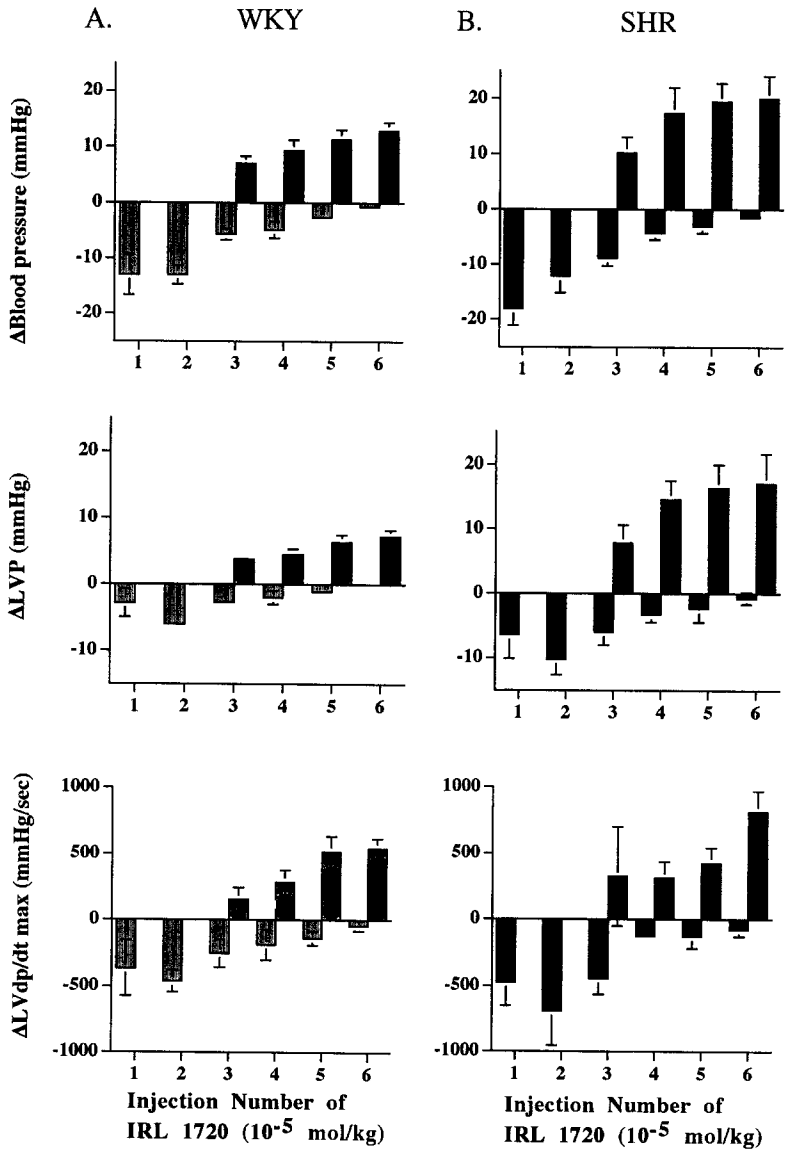

Fig. 4. Effects of six repeated intravenous bolus injections of IRL 1720 (10^{-5} mol/kg). Values are means ± S.E.M. of 10 experiments. See Fig. 3 for further explanation.

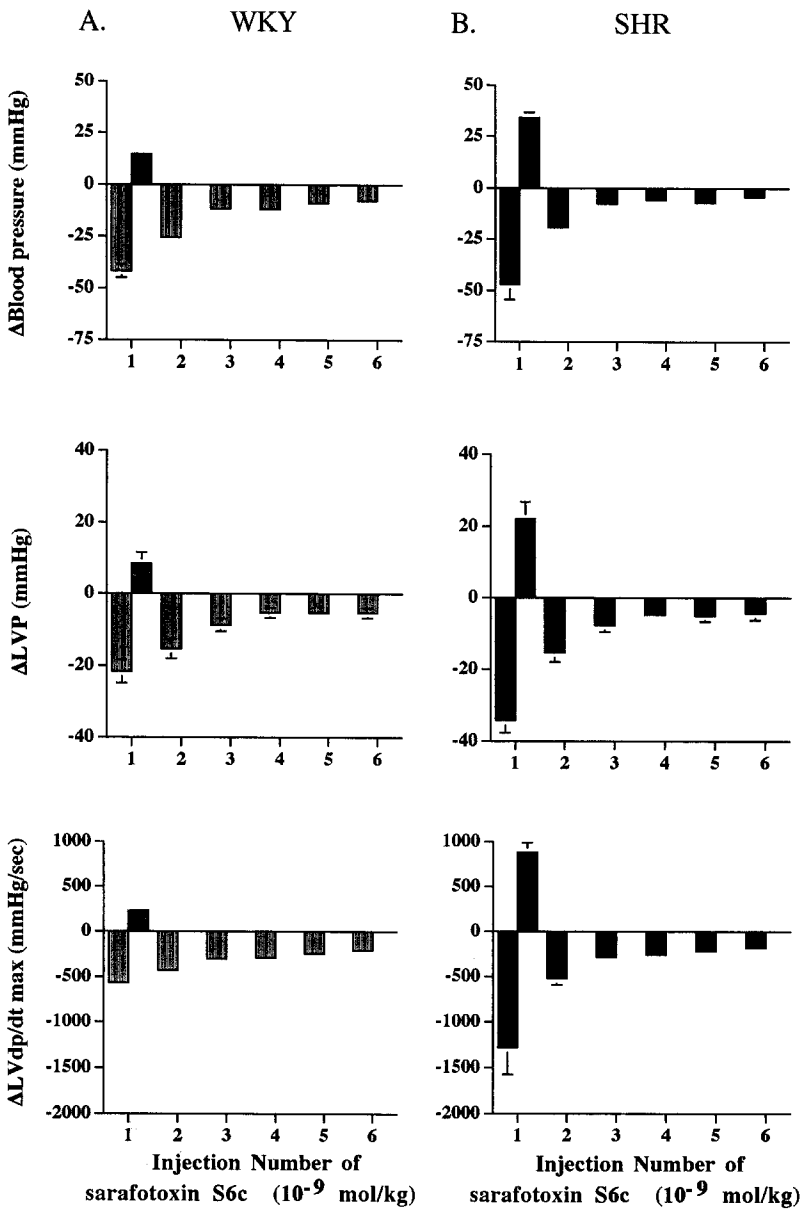

Fig. 5. Effects of six repeated intravenous bolus injections of sarafotoxin S6c (10^{-9} mol/kg). 10 min elapsed between two doses. Values are means ± S.E.M. of five experiments. See Fig. 3 for the explanation of abbreviations.

the second injection of sarafotoxin S6c induced only a short-lasting decrease. Moreover, the depressor response was reduced with the repeated injections of sarafotoxin S6c in both Wistar-Kyoto rats (Fig. 5A) and spontaneously hypertensive rats (Fig. 5B).

3.5. Effects of IRL 1720 or sarafotoxin S6c after repeated injections of IRL 1720 or sarafotoxin S6c

After the five repeated injections of 10^{-5} mol/kg IRL 1720, the intravenous bolus injection of 10^{-6}

Table 3
Effects of intravenous bolus injection of IRL 1720 (10^{-6} mol/kg) or sarafotoxin S6c (10^{-9} mol/kg) after five repeated injections of IRL 1720 (10^{-5} mol/kg) on cardiovascular functions in anesthetized spontaneously hypertensive rats

	ΔBP (mm Hg)		ΔLVP (mm Hg)		$\Delta LVdp/dt_{max}$ (mm Hg/s)	
	Decrease	Increase	Decrease	Increase	Decrease	Increase
IRL 1720 (10^{-6} mol/kg)	−3.3 ± 0.8	8.2 ± 3.4	−2.5 ± 1.2	6.1 ± 3.3	−81.5 ± 15.7	85.7 ± 62.3
Sarafotoxin S6c (10^{-9} mol/kg)	−16.2 ± 7.8	21.3 ± 5.8	−15.3 ± 5.8	18.9 ± 2.8	−383.4 ± 97.3	701.5 ± 176.7

ΔBP: changes of mean arterial pressure; ΔLVP: changes of left intraventricular pressure; $\Delta LVdp/dt_{max}$: changes of maximum differential value of left intraventricular pressure. Values are means ± S.E.M. of three experiments.

mol/kg IRL 1720 to the spontaneously hypertensive rats induced a biphasic response consisting of an initial short-lasting decrease that was smaller than that observed without the IRL 1720 pretreatment, followed by a sustained increase in these parameters that was not observed without the IRL 1720 pretreatment (Table 3). The injection of 10^{-9} mol/kg sarafotoxin S6c after five injections of 10^{-5} mol/kg IRL 1720 also induced a biphasic response (Table 3). The initial short-lasting decrease was weaker than those to the first injection of sarafotoxin S6c without the IRL 1720 pretreatment. In contrast, the subsequent increments were similar to those without the IRL 1720 pretreatment. The intravenous bolus injection of 10^{-5} mol/kg IRL 1720 had almost no effect on cardiovascular responses after five intravenous injections of 10^{-9} mol/kg sarafotoxin S6c ($n = 3$, data not shown).

4. Discussion

In the first series of experiments, we applied the stepwise increasing doses of a selective endothelin ET_B receptor agonist, IRL 1720, to anesthetized spontaneously hypertensive rats and Wistar-Kyoto rats by repeating the single intravenous bolus injection at 30–60-min intervals and measured the cardiovascular parameters including blood pressure, left intraventricular pressure and left intraventricular dp/dt_{max}. Injections of 10^{-8} mol/kg and 10^{-7} mol/kg IRL 1720 were almost ineffective. However, 10^{-6} mol/kg induced a transient cardiovascular depressor effect. Higher concentrations (3×10^{-6} and 10^{-5} mol/kg) showed similar effects to those of 10^{-6} mol/kg IRL 1720.

To know if the endothelin receptors were desensitized, the same concentration (10^{-6} or 10^{-5} mol/kg) of IRL 1720 was repeatedly applied to spontaneously hypertensive rats and Wistar-Kyoto rats. When 10^{-6} mol/kg IRL 1720 was injected for the first time, only a small decrease in the cardiovascular parameters was induced. However, the second injection induced greater depressor effects. It has been shown that the systemic depressor effects of endothelin are mediated by stimulation of the endothelin ET_B receptor in vascular endothelium (Karaki et al., 1993a, b, 1994), resulting in a release of endothelium-derived relaxing factors (De Nucci et al., 1988; Sakata et al., 1989). In isolated blood vessels, it has been widely observed that the endothelium-dependent relaxation is augmented by repeated stimulation of the endothelium. The augmentation of the depressor effect of 10^{-6} mol/kg IRL 1720 may be due to a similar mechanism. The first and the second injections of a higher dose of IRL 1720 (10^{-5} mol/kg) also induced small decreases in the cardiovascular parameters. However, the third injection produced a biphasic response consisting of a much smaller decrease followed by an increase in the cardiovascular parameters. The initial decrease was reduced, whereas the following increase was enhanced by the repeated injection of IRL 1720. Since IRL 1720 binds selectively to the endothelin ET_B receptor (James et al., 1993) and since the pressor effects of IRL 1720 were not inhibited by the endothelin ET_A receptor antagonist, FR139317, these effects seem to be mediated by the endothelin ET_B receptor. These results suggest that repeated injections of higher concentrations of IRL 1720 (10^{-5} mol/kg) may desensitize the depressor effect and reveal the pressor effect that had been hidden by the depressor effects. This possibility was supported by the fact that, after repeated injections of 10^{-5} mol/kg IRL 1720, a monophasic depressor effect of 10^{-6} mol/kg IRL 1720 changed to a biphasic effect consisting of an attenuated depressor effect followed by a pressor effect that was not observed before the repeated injections of 10^{-5} mol/kg IRL 1720.

Because the systemic vasodilator responses to acetylcholine were not altered after desensitization to IRL 1720, and because the vasorelaxant responses to substances including acetylcholine and bradykinin that release endothelium-derived relaxing factors are not subject to desensitization (Ignarro et al., 1989), the endothelin ET_B receptor, but not the formation and release of endothelium-derived relaxing factors, seemed to be desensitized. Our results indicated that the endothelin ET_B receptor mediating endothelium-dependent relaxation in the isolated rat aorta was strongly desensitized by IRL 1720 whereas the endothelin ET_B receptor mediating contraction in the isolated rabbit saphenous vein was desensitized by 300 nM sarafotoxin S6c but not by 1 μM IRL 1720 (unpublished observation).

To further examine the effects of repeated stimulation of the endothelin receptor, the effects of repeated injections of another selective endothelin ET_B receptor agonist, 10^{-9} mol/kg sarafotoxin S6c (Williams et al., 1991), were investigated. The first injection of sarafotoxin S6c induced a large decrease followed by an increase in the cardiovascular parameters, as has been reported by Clozel et al. (1992), whereas the second injection produced only a decrease. The depressor responses became smaller with the repeated injection of sarafotoxin S6c. These results are similar to those obtained for repeated bolus intravenous injection of endothelin-3 in the cat (Le Monnier de Gouville et al., 1990).

The depressor effects of 10^{-6}–10^{-5} mol/kg IRL 1720 were weaker than those of 10^{-9} mol/kg sarafotoxin S6c although binding assay in swine lung membrane showed that the affinities of these agonists for the endothelin ET_B receptor are similar (James et al., 1993). One of the possible explanations for this discrepancy is that the endothelin ET_B receptor subtype

in the swine lung membrane is different from that in the rat cardiovascular system. Another possibility is that sarafotoxin S6c is a full agonist whereas IRL 1720 is a partial agonist of the endothelin ET_B receptor. The latter possibility is supported by the fact that the potency of IRL 1720 to induce the endothelium-dependent relaxation of isolated rat aorta was approximately 100 times less than that of endothelin-3 and sarafotoxin S6c (unpublished observation). The fact that repeated injections of 10^{-5} mol/kg IRL 1720 attenuated the depressor effect of sarafotoxin S6c and that repeated injections of sarafotoxin S6c completely inhibited the effects of 10^{-5} mol/kg IRL 1720 also suggests that these two agonists bind to the same endothelin ET_B receptor and that the efficacy of sarafotoxin S6c is greater than that of IRL 1720.

In contrast to the effects of IRL 1720, the pressor effect of sarafotoxin S6c was observed only once and the second injection induced only a depressor effect of reduced magnitude. However, the results obtained with IRL 1720 suggested that the pressor endothelin ET_B receptor is less tachyphylactic than the depressor endothelin ET_B receptor. One of the possible explanations is that sarafotoxin S6c stimulates the contractile endothelin ET_B receptor more strongly than IRL 1720 and thus desensitizes this receptor as well as the depressor endothelin ET_B receptor. In fact, sarafotoxin S6c induced a stronger desensitization than IRL 1620, an analog of IRL 1720, in the isolated rabbit saphenous vein (Sudjarwo et al., 1994). Another possibility is that the pressor effect is the result of a baroreflex to the depressor effect and, therefore, the decrease in the depressor effect also attenuated the pressor effect. If this is the case, it is also suggested that the pressor endothelin ET_B receptor is stimulated by IRL 1720 but not by sarafotoxin S6c. However, this possibility is not likely because the heart rate did not change with both IRL 1720 and sarafotoxin S6c. Moreover, sarafotoxin S6c showed a stronger contractile effect in the isolated vein than IRL 1720 (unpublished observation).

The characteristics of IRL 1720-induced cardiovascular responses were qualitatively similar for both spontaneously hypertensive rats and Wistar-Kyoto rats. However, both depressor and pressor effects of IRL 1720 were more potent in spontaneously hypertensive rats than in Wistar-Kyoto rats. Recently Yokokawa et al. (1994) have shown that the endothelin-3-induced increases in cytosolic free Ca^{2+} level and inositol 1,4,5-trisphosphate level in endothelial cells of spontaneously hypertensive rats are greater than those in endothelial cells of Wistar-Kyoto rats. Furthermore, Batra et al. (1993) have shown that sarafotoxin S6c evokes much larger increases in intracellular Ca^{2+} in the aortic smooth muscle cells isolated from spontaneously hypertensive rats than in the same cells isolated from Wistar-Kyoto rats. These results suggest that the potent cardiovascular responses to IRL 1720 in spontaneously hypertensive rats are due to increased endothelin receptors and/or signal transduction system in the endothelial and smooth muscle cells in spontaneously hypertensive rats.

In summary, our results suggest that the tachyphylactic endothelin ET_B receptor mediates a cardiovascular depressor effect whereas the less-tachyphylactic endothelin ET_B receptor mediates pressor responses to IRL 1720 in the anesthetized rat. These results also support the hypothesis that the pressor response to endothelin is not mediated entirely through activation of the endothelin ET_A receptor.

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