Changes in the Endothelin-1-induced Contraction of Aorta in Streptozotocin-induced Diabetic Rats

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Vascular diseases are significant complications of diabetes mellitus (DM), and the endothelial cells may play a pivotal role in the development of vascular disease in DM. Endothelin-1 (ET-1) released from endothelium is a potent vasoconstrictor peptide and circulating level of ET-1 is increased in a variety of disease states. The purpose of this study was to determine the changes of responsiveness to ET-1 in DM, and we experimented on the changes in the ET-1-induced contraction, levels of nitrite and lipid peroxidation, and ET-1 immunoreactivity in aorta from streptozotocin-induced DM rats. DM was induced by single injection of streptozotocin (55 mg/kg, i.p.). The immunoreactive ET-1 levels in endothelial layer of thoracic aorta were much higher in DM rats than control rats. Nitrite in tissue homogenate was decreased and plasma nitrite was increased in DM rats. Malondialdehyde (MDA) was significantly increased in DM rats and cGMP was not significantly different between control and DM rats. ET-1 produced concentration-dependent contractile responses that are significantly attenuated in DM rats compared to controls. In the presence of selective ET\textsubscript{A} receptor antagonist BQ610, the maximum contraction was decreased and the concentration ratios for BQ610 yielded pA\textsubscript{2} values of 7.3 (slope, 0.65) in control rats, whereas BQ610 had no antagonistic effect on ET-1-induced contraction in DM rats. However, pretreatment with BQ788, an ET\textsubscript{B} receptor antagonist, maximum response was decreased and the dose-response curves for ET-1 were shifted to the right in both groups and pA\textsubscript{2} values were 7.9 and 7.7 (slope, 1.05 in control and DM rats), respectively. IRL 1620 and sarafotoxin S6c, ET\textsubscript{B} agonists, induced relaxation in control rats but not in DM rats. These results indicate that endothelial cell dysfunction and enhanced immunoreactivity of ET-1 have been found in DM rat and ET-1-induced contraction was attenuated in DM rat. These attenuated responses might be at least in part caused by the alteration of ET\textsubscript{A} receptor properties (e.g. desensitization), and partly related with an alteration in intracellular mechanism for contraction to ET-1.

Key Words: Diabetic mellitus, Endothelin-1, Endothelial dysfunction

INTRODUCTION

Diabetes mellitus (DM) is a contributing factor in the pathogenesis of many vascular diseases and increases the physiological change that increase the rate of death (Ruderman et al, 1992). Especially, DM causes an imbalance of protein and lipid catabolism and induces the hypercholesterolemia, atherosclerosis, hypertension and various cardiovascular diseases (Lavy et al, 1973; Cohen et al, 1983; Wolf et al, 1983; Aboot et al, 1987). The mechanism of DM complication remains largely unknown. In recent study, vascular segments from DM animals demonstrate impaired endothelium-dependent relaxation to receptor mediated relaxants (Oyama et al, 1986; Meraji et al, 1987; Tesfamariam et al, 1989), impaired responses to constrictor and relaxant by disproportion between constrictor and relaxant that are released from endothelial cells (Furchgott et al, 1989; Moncada et al, 1991; Vanhoutte et al, 1991), abnormal patterns of calcium mobilization (Wang et al, 1998), increased platelet adhesion and coagulation (Mayne et al, 1970; Heath et al, 1971; Sagel et al,
1975), structural and morphological changes in endothelial cells (Colwell et al, 1979; Moore et al, 1985), loss of endothelial cells (Lorenzi, 1992), and increased oxidized low-density lipoprotein by lipid peroxidation (Makita et al, 1996). Endothelial cell damage or dysfunction is widely regarded as a critical initiating factor in the development of atherosclerosis and many other vascular diseases. Endothelial cells play a crucial role in the control of blood vessel tone, particularly as mediators of vasodilation and vasoconstriction to a number of vasoactive substances including endothelium-derived contracting factor (EDCF), endothelium-derived relaxing factor (EDRF), and endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Yanagisawa et al, 1988; Angus & Cocks, 1989; Furchgott & Vanhoutte, 1989).

Endothelin-1 (ET-1), a vasoconstrictor peptide secreted from endothelial cells, is thought to play a role in a number of vascular diseases and vasospasm (Yanagisawa et al, 1988). Endothelin (ET) consists of 21 amino acid residues and 2 disulfide bridges and its family consists of three isoforms, namely ET-1, ET-2 and ET-3. These have been shown to exert a wide variety of biological actions (Higgins et al, 1993), and all of which are thought to be mediated by ET receptors (Itoh et al, 1988; Yanagisawa et al, 1988; Inoue et al, 1989). Contractile responses mediated by ET<sub>A</sub> and ET<sub>B</sub> receptors are located on smooth muscle (Maggi et al, 1989; Warner et al, 1993; Battistini et al, 1994a, b). In recent study, plasma ET-1 levels are increased and ET-1-induced contractions are attenuated in patients with DM (Fulton et al, 1991; Hodgson & King, 1992). Also, endothelial cell cultured in high glucose level condition decreased of the release of ET-1 (Hattori et al, 1991), and ET-1-induced contraction was increased. Based on these, it is highly suggested that ET-1 is related in DM complication.

The purpose of this study was to determine the role of ET-1 in DM vascular complication. For this purpose, we determined the change in immunoactivity of ET-1 in thoracic aorta, and observed changes in ET-1-induced contraction and effects of various ET receptor antagonists and agonists. Furthermore, we examined the changes in sensitivity of ET receptor to ET-1.

**METHODS**

*Induction of experimental diabetes*

Male Sprague-Dawley rats, weighing 300-350 g were treated with streptozotocin (55 mg/kg, i.p.) freshly dissolved in a 0.1 M citrate buffer. One week later, the blood glucose of DM rats was checked with glucometer and only those animals that blood glucose was greater than 200 mg/dL after feeding were selected.

Age-matched control rats were treated with the vehicle (citrate buffer, i.p.) and were used 4 weeks later. All animals were given free access to food and water.

*Immunohistochemistry*

Control and DM rats were anesthetized with pentotal sodium (50 mg/kg, i.p.) and their blood samples for measurement of plasma lipid were collected. Thoracic aortas were carefully isolated, removed from open-chest animals, and placed physiological salt solution (PSS). The aortic segments were carefully cleaned of fat and loose connective tissue and used in immunohistochemistry and in vitro experiments including the tension developments, the measurement of nitrite and malondialdehyde (MDA) levels.

The aorta was placed in 0.01 M picric acid and 2% paraformaldehyde mixture (in 0.1 M sodium phosphate buffer) for the fixation. Then, aorta was dehydrated by immersion in a 7.5%, 15%, 30% sucrose solution (in 0.1 M sodium phosphate buffer) sequentially and frozen after being imbedded in OCT compound (Tissue-Tek, Miles Scientific Inc.). The tissues were serially cut in 5 μm thickness. The sections were mounted on poly-L-lysine coated slide glass and air-dried overnight. The sections were placed in cold aceton for 20 min for fixation and air-dried more than 1 hour. Sections were exposed to 0.3% hydrogen peroxide solution (Junsei Chemical Co.) for 20 min to inhibit the endogenous peroxidase activity and washed three times in PBS at 10-minute intervals. The sections were sequentially exposed to 2% bovine serum albumin (BSA, blocking antibody) for 1 hour then to ET-1 antiserum (mouse monoclonal IgG anti ET-1, Oncogene research products, 1: 400) diluted in buffer (0.02 M PBS contained 0.05% BSA) for overnight. They washed three times in PBS at 10-minute intervals and were incubated biotinylated goat anti-mouse IgG (Oncogene research products, 1: 1000) for 2 hours and washed by the same method. They were incubated in Avidin and biotinylated horseradish peroxidase macromolecular complex (Vector Laboratories, Inc. Vectastain Elite ABC
kit) for 1 hour and washed three times in PBS at 10-minute intervals. Peroxidase activity was visualized by incubation in the solution of diaminobenzidine (DAB) substrate (Vector Laboratories, Inc.). The sections were dehydrated in graded alcohols and xylene and coverslipped with malinol (Muto Pure Chemicals Ltd.).

**Measurement of plasma lipids**

Blood samples were centrifuged (Beckman, Model TJ-6 Centrifuge) at 550 g for 10 min and supernatant was kept at –72°C before the experiment. Total cholesterol, triglyceride, and high density lipoprotein (HDL) were measured with analyzer (Olympus, Model AU5200) by enzymatic-colorimetric method.

**Measurement of nitrite and lipid peroxidation level**

The aortic segments obtained from control and DM rats were isolated and stored immediately in liquid nitrogen and the frozen segments were homogenated by homogenizer (Brinkmann, Kinematiza CH-6010 KRIENS-LU) in 5 volumes of weigh 0.1 M phosphate buffer. The homogenates were centrifuged (1,500 g for 20 min) and measured protein content by Bio-Rad protein assay method (Bradford, 1976) with supernatants.

**Measurement of nitrite**

Griess reagent (800 µl) was added to equal volume of plasma sample and tissue homogenate, respectively, and placed at 25°C for 10 min. The absorbance of the color of the product dye at 540 nm was measured by spectrophotometer (Shimadzu, UV-1201). Standard curve was made by the same method with sodium nitrite solution (Green et al, 1982; Durante et al, 1991).

**Measurement of lipid peroxidation level**

We pipetted 3 ml of 0.05 N HCl into a glass-stoppered, brown-colored, centrifuge test tube and added 0.3 ml of a sample to be examined (thoracic aorta tissue homogenate and standard solution). We added 1 ml of 0.67% (v/v) trichloroacetic acid (TCA) solution, which was freshly prepared and mixed very well, and heated the mixture at 95°C exactly for 30 min in water bath and cooled immediately with tap water. We added 4 ml of methanol/n-butanol (3 : 17) mixture and mixed vigorously and spunned 1,500 g for 20 min at slightly lower temperature than room temperature. Then we measured the absorbance of the upper (butanol) layer at 535 nm. Standard curve was made by the same method with MDA solution (Jose & Slater, 1972).

**Measurement of cGMP**

The supernatants were removed and washed out 4 times with 5 vol diethyl ether. The remaining aqueous phase was dried under a stream of N₂. cGMP was measured in acetylated samples with a kit from Amersham International.

**Measurement of isometric tension by ET-1**

Thoracic aorta was placed in a wax block containing oxygenated PSS, and fat and connective tissue were removed. Aortic segments were cut into ring (2~3 mm) and mounted on parallel wires in 5 ml muscle chamber, which was thermoregulated to 37°C. The medium (PSS) was consisted of NaCl 130, KCl 4.7, NaH₂PO₄ 1.18, MgSO₄ 1.17, CaCl₂ 1.6, NaHCO₃ 14.9 and dextrose 5.5 mM and maintained at pH 7.4 with 95% O₂ - 5% CO₂. Rings were stretched to optimal resting tensions of 2 g. Isometric tension was measured using a Polygraph (Grass Instrument Co., 7 E) and force-displacement transducer (Grass Instrument Co., FT03). Following the equilibrated period, the rings were exposed to 60 mM KCl and obtained contractile response. Concentration-response curves were expressed as a percentage of the contractile response elicited by 60 mM KCl (Emax). In the relaxation response, the rings were precontracted with 10~30 nM U46619 and then exposed to IRL 1620 and sarafotoxin S6c, ET₁ receptor agonists. The concentration of agonist required to produce a half maximal response, EC₅₀ was obtained from each agonists’ dose-response curves. The relaxation curves were obtained from Emax of U46619.

**Drugs**

Endothelin-1 (human, porcine ET-1) and sarafotoxin S6c were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and succ-[Glu₉,Ala₁₁,₁₅]-endothelin-1 (8-21) (IRL 1620), BQ610, BQ788 were purchased from Peninsula Laboratories INC. (Belmont, CA, U.S.A.). 9,11-dideoxy-9α,11α-methano-epoxy PGF₂₅ (U46619) was purchased from Biomol.
Co. (Biomolecular Research, U.S.A.), IRL 1620, BQ-610, and BQ788 were dissolved in 0.1% trifluoroacetic acid and acetonitrile mixture.

Statistics

Values are expressed as means±S.E.M. Results were statistically evaluated by Student’s t-test for the differences between DM and vehicle groups. P<0.05 was accepted as statistically significant.

RESULTS

The changes of body weight and blood glucose levels by the induction of DM

There was no significant difference in pre-injection weights between DM rats and control rats. However, 4 weeks post-injection, DM rats showed a significant decrease in body weight. Blood glucose levels were significantly increased in DM rat (P<0.001)(Table 1).

| Table 1. Body weight and blood glucose of control and streptozotocin-induced DM rats |
|-----------------|-----------------|-----------------|-----------------|
|                 | Weight (g)      | Blood glucose (mg/dL) |
|                 | 0 weeks | 4 weeks | 0 weeks | 4 weeks |
| Control rats    | 281±9 (12)     | 400±9 (11)     | 147±5 (6)     | 143±9 (5)   |
| Diabetic rats   | 288±10 (12)    | 240±15*** (12) | 129±2 (7)     | 594±40*** (11) |

Numbers in parentheses represent the number of experiments.

***P<0.001 vs. control rats.

Fig. 1. Visualization by immunohistochemistry of the distribution of ET-1 in section of the thoracic aorta obtained from control (A and B) and DM rats (C and D). Immunoreactivity for ET-1 was enhanced in luminal endothelium (arrow) of the artery from DM one, but little staining was seen in control rats. Magnification: ×100 (A and C), ×400 (B and D).
Immunoreactivity of ET-1 by immunohistochemistry

Immunolocalization of ET-1 demonstrated that ET-1 was increased more than control rats compared with DM rats on the luminal side (Fig. 1).

The changes of plasma lipid levels by the induction of DM

Total cholesterol and HDL levels showed no significant difference between DM and control rats. However, triglyceride levels were significantly increased in DM rats (control: 75±12 mg/dL vs. DM: 174±37 mg/dL, P < 0.05)(Fig. 2).

![Fig. 2. Plasma total cholesterol (Ch), triglyceride (TG) and HDL levels in control and DM rats. Results are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. *P < 0.05 vs. control.]

Nitric oxide and MDA contents

The concentration of nitrite in homogenates was significantly decreased in DM rats (P < 0.01) but plasma nitrite was higher than in control rats (Fig. 3).

The concentration of MDA in homogenates was significantly higher than in control rats. Levels were 1.22±0.16 nmol/mg protein and 2.96±0.6 nmol/mg protein, respectively (Fig. 4).

cGMP contents

The cGMP contents in aortic segments and plasma showed no significant difference between DM and control rats (Fig. 5).

![Fig. 3. Nitrite levels in the aortic homogenate (A) and plasma (B) of control and DM rats. Results are expressed as means±S.E.M. of 6~7 experiments. **P < 0.01 vs. control.]

![Fig. 4. Lipid peroxidation levels in the thoracic aortic homogenate of control and DM rats. Results are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. *P < 0.05 vs. control.]

![Fig. 5. cGMP levels in aortic homogenate (A) and plasma (B) of control DM rats. Results are expressed as means±S.E.M. of 6~7 experiments.]

The changes in vascular reactivity by induction of DM

**ET-1-induced contractions**

ET-1 produced concentration-dependent contractions. Maximum contraction was significantly decreased in DM rats; Emax was 62.6 ± 8.5% at maximum dose (30 nM) in this experiments (control rats: 101.2 ± 10.6%, P < 0.05) (Fig. 6). EC50 was increased in DM rats from 1.28 × 10^{-8} M to 2.96 × 10^{-8} M.

**ET-1-induced contraction depends on endothelium**

The contraction of endothelium-intact thoracic aorta was decreased in DM rats but the contractile response of endothelium-denuded thoracic aorta showed no difference between control and DM rats (Fig. 7).

**Effects of ET receptor antagonists pretreatment on ET-1-induced contraction**

ET-1-induced contraction was attenuated by pretreatment with BQ610, a selective ETα receptor antagonist not in DM rats but in control rats (Fig. 8A). Schild plot for BQ610 on contraction induced by ET-1 in control rats showed partial competitive inhibition (slope 0.65), but in DM rats it didn’t show the same effect (slope 0.24). pA2 was decreased to 7.3 and 6.8, respectively (Fig. 8B). ET-1-induced contraction was attenuated by pretreatment with BQ788, a selective ETβ receptor antagonist, in DM and control rats (Fig. 9A). Schild plot for BQ788 on contraction induced by ET-1 in control and DM rats

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**Fig. 6.** Dose-dependent contraction to ET-1 in thoracic aorta from control and DM rats. Results are expressed as means ± S.E.M. of 10 experiments. *P < 0.05; **P < 0.01 vs. control.

**Fig. 7.** Contraction to ET-1 (30 nM) in thoracic aorta with and without endothelium from control and DM rats. Each bar represents mean ± S.E.M. of 10–13 experiments. *P < 0.01 vs. control. ED (+): with endothelium; ED (−): without endothelium.

**Fig. 8.** Effect of BQ610, a selective ETα receptor antagonist on ET-1-induced contraction (A) and Schild plot for BQ610 on contraction induced by ET-1 (B) in thoracic aorta from control and DM rats. Results are expressed as means ± S.E.M. of 6–12 experiments. DR is dose ratio. pA2: The logarithm of the antagonist concentration that shifts dose-response to the right by a factor of 2. *P < 0.05; **P < 0.01; ***P < 0.001 vs. ET-1 alone.
showed competitive inhibition (slope 1.05). Also pA₂ was not different between control and DM rats (Fig. 9B).

**Effects of ET receptor agonists on ET1-induced contraction**

The rings were precontracted with 10–30 nM U46619 and then exposed to IRL 1620 and sarafotoxin S6c, ET₁ receptor agonists. In control rats, they produced contractile response but in DM rats, they showed relaxation response (Fig. 10).

**DISCUSSION**

It is well known that DM causes various cardiovascular complications such as hypercholesterolemia, atherosclerosis, hyperension and various cardiovascular diseases (Lavy et al, 1973; Cohen et al, 1983; Wolf et al, 1983; Aboot et al, 1987). DM induced both macroscopic and microscopic structural and morphological changes including increased proliferation of arterial smooth muscle (Beisswenger et al, 1970; Kilo et al, 1972; Johnson et al, 1982), decrease of microvascular density (Bohlen & Niggl, 1979), metamorphosis of endothelial cell layer (Colwell et al, 1979; Moore et al, 1985), increased platelet adhesion and coagulation (Mayne et al, 1970; Heath et al, 1971; Sagel et al, 1975), increased permeability of endothelium, and changes in relaxation responses (Oyama et al, 1986; Meraji et al, 1987; Wakabayash et al, 1987; Durante et al, 1988). Also, recent study showed impaired responses to constrictor and relaxant by disproportion between constrictor and relaxant that were released from endothelial cells (Furchgott et al, 1989; Moncada et al, 1991; Vanhoutte et al, 1991), and abnormal patterns of calcium mobilization (Wang et al, 1998).

Immunolocalization of ET-1 demonstrated that ET-1 expression was increased in DM rats on the luminal side, whereas ET-1 induced contractions were attenuated by DM. Total cholesterol and HDL levels showed no significant differences between DM and control rats. However, triglyceride levels were significantly increased in DM rats. The concentration of nitrite in homogenates was significantly decreased in DM rats, but on the contrary plasma, nitrite levels were increased by induction of DM. The concentration of MDA in DM homogenates was significantly higher than control and the cGMP contents in aortic segments and plasma showed no significant difference between DM and control rats. Based on these results, we examined the mechanisms of at-
tenuated ET-1-induced contraction in DM rats.

In the activation of elevated glucose levels in DM, the release of ET from endothelial cells was increased (Fulton et al, 1991; Hodgson & King, 1992) and immunoreactivity of ET-1 was increased, too (Chakrabarti & Sima, 1997). Our results were confirmed by immunohistochemistry. ET-1 stimulated the hydrolysis of the phosphatidyl 4,5-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG), and increased DAG activated the protein kinase C (PKC) that induced the contraction (Ohlstein et al, 1989; Neylon, 1999). Then, desensitization of PKC by pretreatment of PMA produced attenuated contractile response (Robert et al, 1989; Resink et al, 1990).

In this study, ET-1-induced contractions were attenuated in DM rats. This suggests that, in DM rats, the attenuated ET-1-induced contraction was caused by elevated ET-1-induced catabolism of PIP2 to DAG. And then enhanced be able to DAG stimulate PKC, and finally caused desensitization of PKC. Also, Our data that attenuated ET-1-induced contraction in DM rats was restored in endothelium-denuded thoracic aorta strongly suggest that attenuated ET-1-induced contraction in DM rats was caused by endothelial cell damage.

ET receptors consists of ET<sub>A</sub> receptor and ET<sub>B</sub> receptor (Sakurai et al, 1990). ET<sub>A</sub> receptors are located on smooth muscle. ET<sub>B</sub> receptors are located on smooth muscle and endothelial cells (Maggi et al, 1989; Warner et al, 1993; Battistini et al, 1994a, b). Contractile responses are mediated by ET<sub>A</sub> and ET<sub>B</sub> receptors located on smooth muscle (Cardell et al, 1992; Martine et al, 1992; Graham et al, 1995), whereas relaxation responses are mediated by ET<sub>B</sub> receptors which are located on endothelial cells (Takayanagi et al, 1991; Martine et al, 1992; Alcock et al, 1995). Based on this, we examined the ET receptor subtypes that mediated the ET-1-induced contraction by pretreatment with selective ET receptor subtype antagonists, BQ610 and BQ788. Under the pretreatment with BQ610, selective ET<sub>A</sub> receptor antagonists, slopes were 0.65 in control and 0.24 in DM rats. In addition, pA<sub>2</sub> was decreased from 7.3 in control to 6.8 in DM rats. BQ610 showed partially competitive inhibition in control but not in DM rats. These results indicate that DM caused changes in sensitivity of ET<sub>A</sub> receptor to ET-1. On the other hand, ET-1-induced contraction was attenuated by pretreatment with BQ788, a selective ET<sub>B</sub> receptor antagonist in DM and control rats respectively. Based on these results, It is likely that ET-1-induced contraction was also mediated by ET<sub>B</sub> receptors which are located on smooth muscle. Furthermore, Schild plot for BQ788 on contraction induced by ET-1 in control and DM rats showed competitive inhibition and pA<sub>2</sub> showed no difference between control and DM rats. These results provide that attenuated ET-1-induced contraction in DM rats was not caused by alteration in properties of ET<sub>B</sub> receptors. In the present study, the effect of ET<sub>B</sub> receptors located on endothelial cells and smooth muscle was determined by IRL 1620 and sarafotoxin S6c, ET<sub>B</sub> receptor agonists (Eglezos et al, 1993; Karaki et al, 1993). In the thoracic aorta from control rats, they produced contractile response, whereas in DM rats they showed relaxation response that was precontracted with 10—30 nM U46619. These results suggest that ET<sub>B</sub> receptor-mediated vasodilation ability was damaged by induction of DM, and it might be indirect evidence of endothelial dysfunction.

Increased oxidized low-density lipoprotein by lipid peroxidation (Makita et al, 1996) caused endothelial cell damage and induced atherosclerosis. Our results showed increase of MDA level in tissue homogenates from DM rats. From this evidence, we can not exclude the possibility that increased MDA is also able to cause change in vascular reactivity to ET-1 to DM.

The concentration of nitrite in homogenates was significantly decreased in DM rats but plasma nitrite was higher than control rats. These results may be caused by decreased activity of endothelial nitric oxide synthase (eNOS) and increased activity of inducible nitric oxide synthase (iNOS) (Jang et al, 1999). It is well known that NO produced by NOS stimulates guanylate cyclase and guanylate cyclase produces cGMP. In our result, cGMP content in aortic segments and plasma were not significantly different between DM and control rats. This may be caused by counterbalance from increased NO production by increased activity of iNOS (George, 1990) and decreased NO production by decreased activity of eNOS (Makita et al, 1996). Also, the probability of increased NO production by iNOS activity related to attenuated ET-1-induced contraction is also considerable.

In conclusion, ET-1 plays a role in the regulation of vascular tone in rats via ET<sub>A</sub> receptor mainly and ET<sub>B</sub> receptor located on smooth muscle. And then, the attenuation of ET-1-induced contraction in DM
was caused by decreased binding site to ET-1 (Nayler et al, 1989) and changed receptor subtype activity (Arai et al, 1990). Furthermore, it was caused by changes of lipid peroxidation, endothelial cell damage and the changes of sensitivity of ET receptors to ET-1.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Korean Research Foundation (1998-021-F00236) and a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-98-M-3-0044).

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