

Original Article

Effect of Hypertension on the Occurrence of Micro-hemorrhage in the Pancreatic Islet of Dahl Salt-sensitive Rats

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Abstract: The effect of hypertension on the occurrence of micro-hemorrhage in the pancreatic islet, known to be observed in Sprague-Dawley (SD) rats spontaneously, and endothelial markers were investigated in male Dahl-Iwai salt-sensitive (DIS, derived from SD rats), salt-resistant (DIR), and SD rats. DIS and DIR rats were fed 8% NaCl-containing diet to induce hypertension, with blood pressure measurement once a week, euthanized at 6, 8, or 12 weeks of age, and subjected to the measurement of plasma nitric oxide (NO) and von Willebrand factor (vWF) concentrations combined with histopathological examinations and immunohistochemical detections of vWF in the pancreas and kidney. As a result, hypertension was observed from 7 through 12 weeks of age in DIS rats. At 12 weeks of age, only DIS rats showed decreased plasma NO and increased vWF, indicating endothelial abnormality in the body. Histopathologically, micro-hemorrhage in the islet was observed with a similar incidence and severity in SD and DIS rats aged 12 weeks, and vWF was immunohistochemically localized in the islet endothelium with similar reactivity between age-matched SD rats. On the other hand, in the kidney, glomerular sclerosis was observed in DIS rats aged 12 weeks and accompanied broad stainability of vWF in the sclerotic glomerulus, including endothelium. In conclusion, there was no enhancement/exaggeration in the micro-hemorrhage in the pancreatic islet of hypertensive DIS rats in comparison with that in SD rats under the present experimental conditions. It is suggested that hypertension is not related to the occurrence of islet micro-hemorrhage, spontaneously observed in SD rats. (DOI: 10.1293/tox.25.155; *J Toxicol Pathol* 2012; 25: 155–161)

Key words: Dahl-Iwai salt-sensitive rat, hypertension, pancreatic islet, endothelium, Sprague-Dawley rat

Introduction

In Sprague-Dawley (SD) rats, some information regarding age-related non-neoplastic lesions in the pancreatic islet has been reported¹⁻³. The initial change is characterized as a leakage of red blood cells from the capillaries and hemosiderin deposition in the center or periphery of the islet. This is firstly observed at 12 weeks of age and develops in an age-dependent manner accompanying inflammatory cell infiltration and/or fibrosis. However, the pathogenesis of this micro-hemorrhage has not been clarified.

Regarding the islet capillary, morphologically remarkable features have been shown in comparison with the acinar capillary. The islet capillary is significantly wider, thinner walled and has many more fenestrations of endothelial cells than those in the acinus^{4,5}. Additionally, the blood flow

perfusion of the pancreatic islet constitutes approximately 10% of the whole pancreatic blood flow, despite the islets contributing only around 1% to the pancreatic volume⁶. These morphologic characteristics of the islet endothelium resemble those of renal glomeruli with a dense network of capillaries, highly permeable endothelial cells, and high blood perfusion.

In the kidney, hypertension-induced vascular abnormality is well known. Systemic hypertension causes endothelial dysfunction, increased reactive oxygen species (ROS) in the glomerulus, and renal insufficiency⁷. Likewise, there is a report that hypertensive Ren-2 transgenic rats showed fibrosis and a positive immunohistochemical reaction for 3-nitrotyrosine, an indirect ROS index, in the pancreatic islet⁸. Based on these reports, it is speculated that both capillaries in the glomerulus and the islet are vulnerable to hypertension-induced endothelial damage because of their morphological resemblance and high blood perfusion. The spontaneous micro-hemorrhage in the islet can be exaggerated by hypertension and following endothelial dysfunction. To the best of our knowledge, the effect of hypertension on the occurrence of spontaneous lesion in the islet histologically has not yet been investigated.

In the present study, we compared the occurrence and incidence of micro-hemorrhage in the pancreatic islet with endothelial damage markers using hypertensive rats, Dahl-

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Iwai salt-sensitive (DIS) rats, derived from SD rats, and naïve SD rats. The kidney was investigated as well.

Materials and Methods

Animals

Thirty each of male Slc:SD, DIS, and Dahl-Iwai salt-resistant (DIR) rats aged 5 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed 2 or 3 animals per wire-mesh cage in an air-conditioned room (temperature, $23 \pm 2^\circ\text{C}$; relative humidity, $55 \pm 20\%$) with a 12-hour light/dark cycle. They were allowed free access to a commercial standard diet containing 0.19% of NaCl (F-2, Funabashi Farm Co, Chiba, Japan) and chlorinated tap water during the experimental period, including a quarantine and acclimation period of 1 week. From 6 through 12 weeks of age, DIS and DIR rats were fed 8% NaCl diet (57BQ, Japan SLC, Inc.) to induce hypertension. The other ingredients, except for NaCl, of these two diets were similar, and the percentage of NaCl and feeding period were consistent with those reported by Rapp *et al.* and Yamazaki *et al.* as inducing vascular lesions in a variety of organs/tissues^{7,9}. SD rats were continuously fed the standard diet to reproduce micro-hemorrhage in the islet as previously reported^{2,3}.

Experimental design

Figure 1 shows details of the study design. The animals were divided into 3 groups of 10 animals each for euthanasia at 6, 8, and 12 weeks of age. All the animals were observed for general conditions once daily on weekdays throughout the experimental period. Body weights were measured weekly for each strain from ages 5 to 12 weeks. Additionally, systolic blood pressure (SBP) and mean blood pressure (MBP) were measured weekly using the tail-cuff method with an automatic sphygmomanometer (BP98A-L, Softron, Tokyo, Japan). Measurement was performed three times each in three animals of the group using the middle of the tail, and means and standard deviations were calculated at each time point.

Laboratory examinations

Under ether anesthesia, 2 mL of blood was collected from the jugular vein of rats at 6 and 12 weeks of age with a disposable syringe, and approximately 1 mL of each sample was transferred to a heparin-coated tube (Becton Dickinson and Company). Obtained plasma from the heparin-added blood was used for nitric oxide (NO, as the sum of nitrate and nitrite in this assay) measurement using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, MI, USA) for detection of endothelial dysfunction. Plasma von Willebrand factor (vWF) was measured by an enzyme-linked immunosorbent assay with a commercial kit (USCN Life Science Inc., Wuhan, China).

Light microscopy

Following blood collection, rats were euthanized by exsanguination under ether anesthesia. The pancreas was

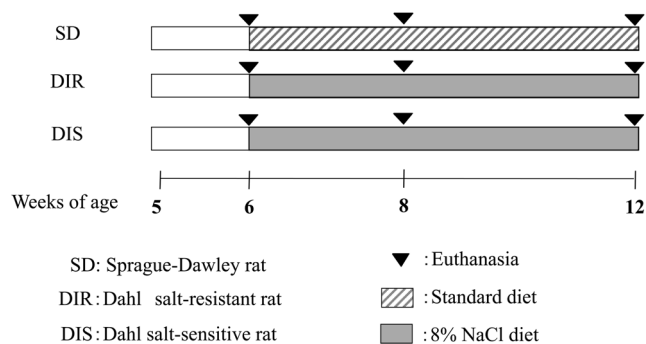


Fig. 1. Study design.

removed and immediately fixed in 10% neutral buffered formalin. The tissues were trimmed into 3 regions, including right (duodenal segment), body (parabiliary and gastric segments), and left regions (splenic segment), embedded in paraffin wax, cut at $4 \mu\text{m}$ in thickness, stained with hematoxylin and eosin (H&E), and examined microscopically. The incidence of rats having lesions in the pancreatic islet in all 3 sections for each age group was recorded. To compare the exact incidence of the lesion, the incidence (percentage) of the islets having the lesion was calculated in the total number of islets on the 3 sections. Additionally, the kidneys from the 12-week-old animals were also collected and examined similarly.

Immunohistochemistry

Immunohistochemical staining for vWF as an endothelial marker was performed in representative sections of the pancreas in 6- and 12-week-old SD and DIS rats. Kidneys of 12-week-old SD and DIS rats were also tested. An immunoglobulin conjugated to a peroxidase-labeled dextran polymer (EnVision, Dako Japan, Tokyo, Japan) was used. In brief, sections were deparaffinized and digested by proteinase K (Millipore, MA, USA) for 8 minutes at room temperature for antigen retrieval. After quenching of endogenous peroxidase with 0.3% H_2O_2 , sections were incubated with a protein block (Dako Japan). Then, sections were incubated with the primary antibody, rabbit anti-human vWF (Dako Japan) for 60 minutes and stained with the EnVision system (Dako Japan). Immunoreactivity was detected by means of horseradish-peroxidase-streptavidin complex using diaminobenzidine chromogen as a marker. The sections were then counterstained with hematoxylin. As negative controls, pancreas and kidney sections untreated with the primary antibody were used.

Statistical analyses

Body weights, SBP, MBP, concentrations of NO and vWF in plasma, and the incidence of islets having the lesion were analyzed using Tukey's multiple comparison test at each week of age to focus on strain differences. Additionally, NO and vWF were also analyzed by Student's *t* test, versus the 6-week-old group in each strain to focus on age-

Table 1. Body Weights in SD, DIR, and DIS Rats

Weeks of age	6	7	8	9	10	11	12
Number of animals	30	20	20	10	10	10	10
Strains							
SD	185.9 ± 8.2	260.4 ± 12.0	312.8 ± 15.8	353.8 ± 15.7	382.4 ± 18.4	401.0 ± 23.0	425.8 ± 27.0
DIR	191.1 ± 22.4	262.0 ± 25.7	314.0 ± 21.3	346.9 ± 20.9	371.5 ± 21.3	393.2 ± 19.3	413.0 ± 19.9
DIS	185.5 ± 6.9	246.6 ± 8.6*	288.2 ± 9.6**	314.4 ± 14.3**	333.1 ± 17.3**	342.2 ± 18.4**	359.3 ± 20.4**

Values are shown with means and standard deviations. * $p < 0.05$, ** $p < 0.01$: Significantly different from the age-matched SD and DIR groups (Tukey's multiple comparison test).

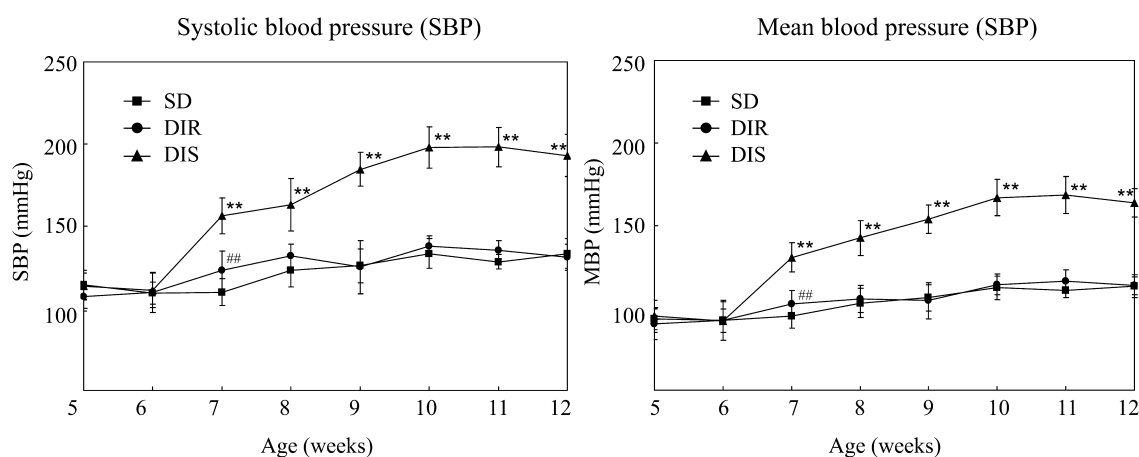


Fig. 2. Systolic and mean blood pressure (SBP and MBP, respectively) in SD, DIR, and DIS rats. Higher SBP and MBP are observed in DIS rats from 7 through 12 weeks of age as compared with age-matched SD and DIR rats. DIR rats show slight increases compared with SD rats only at 7 weeks of age. ** $p < 0.01$: Significantly different from the age-matched SD and DIR groups. ## $p < 0.01$: Significantly different from the age-matched SD groups (Tukey's multiple comparison test).

related changes. The incidences of animals having the lesion in the pancreatic islets were analyzed by χ^2 test. A p value less than 5% (two-tailed) was considered to be significant.

Animal welfare

The experimental protocol was approved by the Ethics Review Committee for Animal Experimental of Daiichi Sankyo Co., Ltd. All experimental procedures were performed in accordance with the "Law Concerning the Protection and Control of Animals" and "Standards relating to the care and management, etc. of experimental animals" in Japan.

Results

Blood pressure and laboratory examinations

Body weights, SBP and MBP in DIS, DIR and SD rats are shown in Table 1 and Fig. 2, respectively. Compared with SD and DIR rats, higher SBP and MBP were obtained in DIS rats from 7 through 12 weeks of age, while DIR rats showed a sporadic increase only at 7 weeks of age. The body weights of the DIS rats were lower but slowly increased during the same period as compared with those of the DIR and SD rats without abnormal clinical signs. NO concentration showed a decrease in DIS rats at 12 weeks of age compared with those in 6-week-old DIS and 12-week-old SD rats (Fig.

3). Additionally, vWF concentration increased in DIS rats at 12 weeks of age compared with that in DIS rats at 6 weeks of age.

Light microscopy

The incidence of lesions in the pancreatic islet is presented in Table 2. The changes were observed as a few erythrocytes considered to have leaked from capillaries with brown pigments, or pigment deposits without erythrocytes in the center/periphery of islets (Fig. 4a). These changes were located in very limited portions of the islet in all animals. The brownish pigments were stained positively with Prussian blue and confirmed to be hemosiderin (data not shown). The lesion was observed at 12 weeks of age in all three strains, and the incidence of animals was similar among them. The incidence of islets having a hemorrhage was very low in all strains, and no significant difference was observed (Table 2). On the other hand, DIS rats occasionally showed inflammatory cell infiltration around the arteries in the pancreas at 12 weeks of age.

In terms of the kidney, sclerotic lesion in the glomerulus was observed in DIS rats at 12 weeks of age, whereas no changes were seen in the SD rats (Figs. 4c and 4e). There was no change in the arteries and arterioles of the kidney in any of the sections examined.

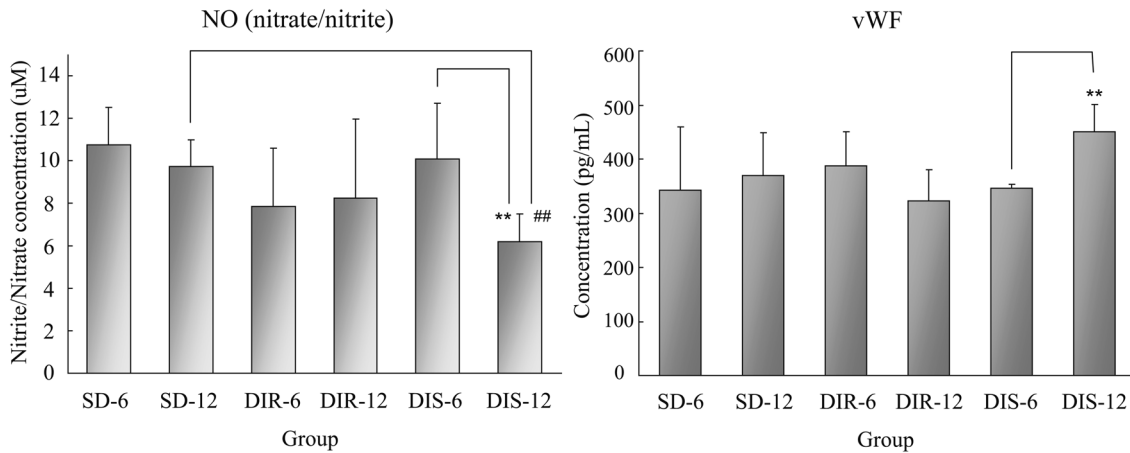


Fig. 3. Plasma concentrations of NO and von Willebrand factor (vWF) in SD, DIR, and DIS rats. ^{##} $p < 0.01$: Significantly different from SD rats at 12 weeks of age (Tukey's multiple comparison test). ^{**} $p < 0.01$: Significantly different from DIS rats at 6 weeks of age (Student's *t* test).

Table 2. Microscopic Incidence of Micro-hemorrhage in the Pancreatic Islet

Weeks of age		6	8	12
Number of animals		10	10	10
Strains	SD	0 ^a	0	6 (1.4 ± 2.7) ^b
	DIR	0	0	5 (0.9 ± 1.3)
	DIS	0	0	8 (1.4 ± 1.0)

^a Number of animals showing micro-hemorrhage. ^b Incidence (%), the number of islets having micro-hemorrhage/the total number of islets on the section. Mean ± standard deviation.

Immunohistochemistry

Immunohistochemistry for vWF revealed a positive reaction in the vascular endothelial cells in the pancreas and kidney. In the pancreas, a positive reaction was obtained in both the islet with and without the micro-hemorrhage. However, its stainability was generally similar, and no difference was seen in the staining intensity between strains and ages (Fig. 4b). In the kidney, a positive reaction was obtained in almost all of the glomerular endothelium in both the 6 and 12 weeks of age groups of SD and DIS rats. In the sclerotic glomerulus of DIS rats at 12 weeks of age, vWF was stained broadly in the sclerotic area, including the endothelium (Figs. 4d and 4f). There was also immunoreactivity in the endothelium of large arteries and arterioles in both organs without a difference in their stainability, including the pancreatic artery, which showed an inflammatory reaction.

Discussion

In the present study, DIS rats showed a clear increase in blood pressure and slight decrease in body weight for 5 weeks, and decreased NO and increased vWF concentrations in plasma at 12 weeks of age. NO is synthesized from molecular oxygen and L-arginine in the endothelial cell. Similarly, vWF is produced in the endothelium, and a de-

creased NO due to endothelial damage causes the release of vWF from Weibel-Palade bodies in the endothelium^{10, 11}. The effect of decreased body weight gain on the alteration of these parameters has not been reported, but endothelial damage in the body was strongly implied.

Histopathological sclerosis in the glomerulus at 12 weeks of age was consistent with the previous report regarding hypertension-induced glomerular endothelial changes in DIS rats fed an 8% NaCl-containing diet for 4 weeks¹². Moreover, broad immunoreactivity in vWF was obtained in the sclerotic glomerulus of this group. While all the organs and vasculatures were not examined in the present study, these results suggested that the decreased/increased endothelial biomarkers in plasma are contributed, at least in part, by the endothelial abnormality in the glomerulus.

On the contrary, no sclerotic/inflammatory changes and only micro-hemorrhage was observed in the islet of all the tested strains at 12 weeks of age, and there was no exaggeration in severity or increased incidence of the lesion. Although DIS rats receiving a high-salt diet are known to show apparent inflammation in the pancreatic arteries¹³, which was consistent with our results, an islet capillary lesion has not been reported. It is suggested that the islet vasculature in the DIS rat has a lower susceptibility to hypertension-induced lesion than that in pancreatic arteries and glomerular vasculature. Since the 8% NaCl diet has been reported to induce 80–100% death after 7 to 8 weeks of feeding, it was difficult to find the consequence of these lesions in older DIS rats^{12, 13}. However, it is possible to obtain a slower development of hypertension and a longer life span by using a diet with a lower NaCl concentration. It has been reported that a diet containing 0.3 or 4% of NaCl delays the occurrence of hypertension and mortality in animals¹². Further investigation including different experimental conditions may be required.

Hypertension-induced endothelial abnormalities have been reported in a variety of blood vessels including arter-

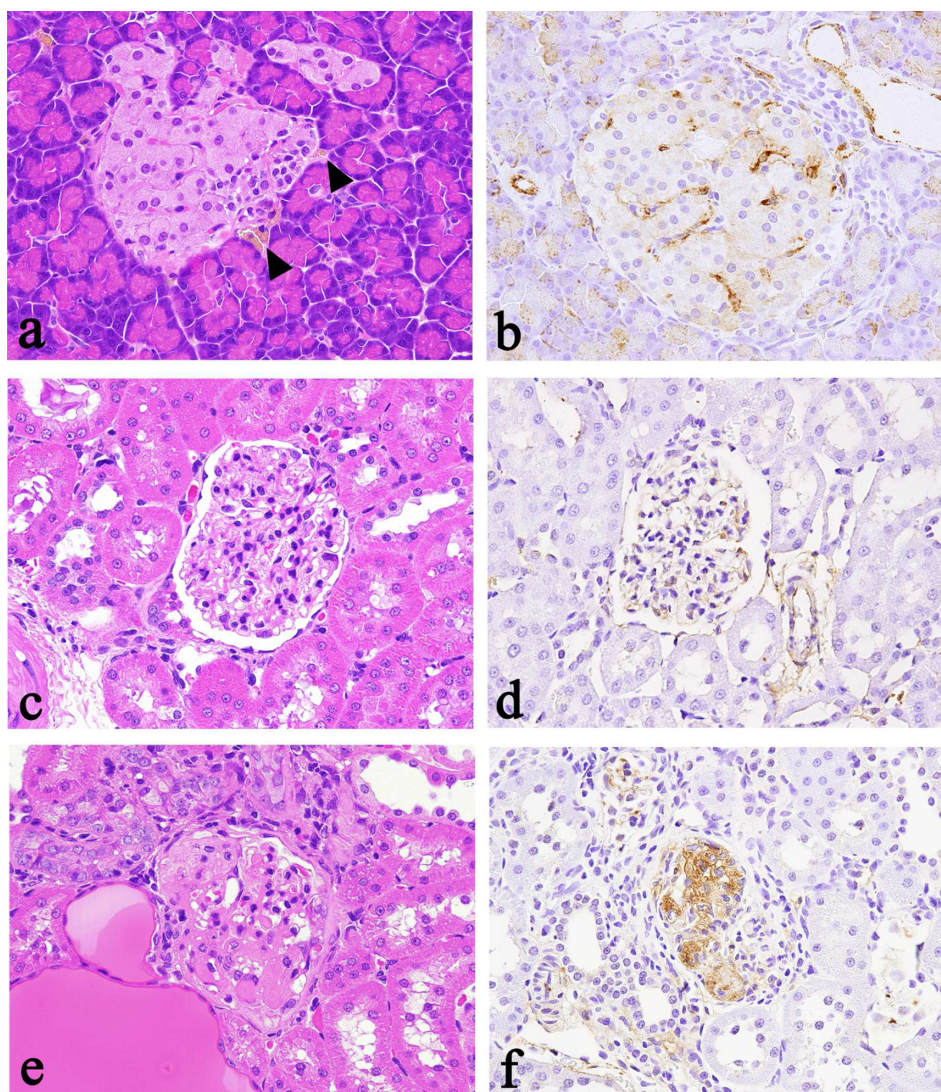


Fig. 4. a) The pancreatic islet in an SD rat at 12 weeks of age. Micro-hemorrhage is observed as a few erythrocytes considered to have leaked from capillaries with brown pigments, or pigment deposits without erythrocytes in the periphery of pancreatic islets (arrowheads). H&E, $\times 600$. b) Immunohistochemistry for vWF in the pancreatic islet. A positive reaction is obtained in the islet endothelium with and without micro-hemorrhage. $\times 600$. c) The glomerulus in the kidney of an SD rat at 12 weeks of age. No abnormal findings are observed. H&E, $\times 600$. d) Immunohistochemistry for vWF in the glomerulus of an SD rat at 12 weeks of age. The stainability in the glomerular endothelium is weak. $\times 600$. e) The glomerulus in the kidney of a DIS rat at 12 weeks of age. A sclerotic lesion is observed in the glomerulus. H&E, $\times 600$. f) Immunohistochemistry for vWF in the glomerulus of a DIS rat at 12 weeks of age. A broad positive reaction is obtained in the sclerotic area including the endothelium. $\times 600$.

ies and capillaries. It is well known that hypertensive endothelial dysfunction is characterized by an imbalance between NO and ROS production from endothelial cells^{9,14,15}. According to data from Satoh *et al.*, hypertensive DIS rats (receiving an 8% NaCl-containing diet for 6 weeks) demonstrated decreased NO and increased ROS in the endothelium of the glomerulus and arterioles in the kidney by *in situ* fluorescent perfusion method, and this accompanied histopathological sclerosis in the glomerulus⁷. The histopathological change in the kidney of the present study is considered to be

a reflection of these findings. However, there have been few reports concerning the pancreatic islet.

As for the islet, the close relationship between hypertension and type 2 diabetes mellitus is widely accepted. Hypertension is a common comorbid condition in diabetes; it has been reported that patients with essential hypertension are more prone than normotensive subjects to develop diabetes¹⁶. In the animal model, impaired endothelium-dependent vasodilation has been shown in diabetic rats induced by alloxan or streptozotocin¹⁷⁻¹⁹. Additionally, there are re-

ports that telmisartan, an angiotensin II blocker, reduced oxidative stress in the islet and prevented the development of diabetes in Spontaneous Diabetic Torii (SDT) rats^{20,21}. Decreased systemic blood pressure possibly corresponds to decreased local blood pressure in the islet and attenuated production of oxidative stress in the islet²⁰. The series of changes in the islet of SDT rats is known to be microvascular hemorrhage/congestion and subsequent inflammation and fibrosis²². While a detailed explanation has not been published, it is possible to consider that the initial injury of the endothelium provokes an increase in oxidative stress and development of morphological changes toward fibrosis in the islet. Although the present study could not enhance the occurrence of islet hemorrhage and fibrosis, the above information is considered to be helpful for exploration of the mechanism of islet hypertension and its influence on the occurrence and development of histological lesions.

Aging is also associated with endothelial dysfunction and reports suggesting age-dependent endothelial changes are available in animals and humans: a study using Wistar-Kyoto (WKY) rats demonstrates WKY rats at 63 weeks of age showed lower NO synthase activity than younger animals²³. In humans, the effects of aging and hypertension on endothelial function have been described²⁴. The authors revealed that elderly normotensives exhibited lower endothelium-dependent relaxation and higher subcutaneous capillary pressure as compared to those in young normotensives, while there were identical degrees of these parameters between elderly normotensives and elderly hypertensives. In this investigation, aging is considered to be more crucial than hypertension for the occurrence of endothelial dysfunction and the increased capillary pressure. In our work, there was no evidence indicating islet-endothelial dysfunction even in DIS rats, whereas the micro-hemorrhage was observed histopathologically. The reason for the occurrence of this micro-hemorrhage was unknown. It is possible that the lesion was too modest to result in detectable changes in vWF and NO in plasma and immunohistochemistry. In SD rats, the incidence of the lesion is extremely rare at an initial observation, occurring in less than 2% of the total number of islets at 12 weeks of age². The exploration of the etiopathogenesis of this lesion is considered to be very difficult. On the other hand, the incidence of the lesion increases drastically at 26 weeks of age, occurring in over 20% of the islets³. There might be a trigger for more obvious lesions, and aging is one of the possible factors. This is an issue to be examined in the future.

In summary, there was no exaggeration of islet micro-hemorrhage even in hypertensive DIS rats, while endothelial dysfunction was detected as changes in plasma endothelial markers and the histopathological findings in the glomerulus and pancreatic arteries. It is suggested that hypertension is not related to the occurrence of this micro-hemorrhage, spontaneously observed in SD rats.

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