Original Article

A Dipeptidyl Peptidase-4 Inhibitor but not Incretins Suppresses Abdominal Aortic Aneurysms in Angiotensin Ⅱ-Infused Apolipoprotein E-Null Mice

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***Aim*: The main pathophysiology of abdominal aortic aneurysm (AAA) considerably overlaps with that of atherosclerosis. We reported that incretins [glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP)] or a dipeptidyl peptidase-4 inhibitor (DPP-4I) suppressed atherosclerosis in apolipoprotein E-null (Apoe−/−) mice. Here we investigated the effects of incretin-related agents on AAA in a mouse model.**

***Methods*: Apoe−/− mice maintained on an atherogenic diet were subcutaneously infused with saline, Ang Ⅱ (2000 ng/kg/min), Ang Ⅱ, and native GLP-1 (2.16 nmol/kg/day) or Ang Ⅱ and native GIP (25 nmol/kg/day) for 4 weeks. DPP-4I (MK0626, 6 mg/kg/day) was provided in the diet to the Ang Ⅱ-infused mice with or without incretin receptor antagonists [(Pro3) GIP and exendin (9-39)].**

***Results*: AAA occurred in 70% of the animals receiving Ang Ⅱ. DPP-4I reduced this rate to 40% and significantly suppressed AAA dilatation, fibrosis, and thrombosis. In contrast, incretins failed to attenuate AAA. Incretin receptor blockers did not reverse the suppressive effects of DPP-4I on AAA. In the aorta, DPP-4I significantly reduced the expression of Interleukin-1β and increased that of tissue inhibitor of metalloproteinase (TIMP)-2. In addition, DPP-4I increased the ratio of TIMP-2 to matrix metalloproteinases-9.**

***Conclusions*: DPP-4I, MK0626, but not native incretins has protective effects against AAA in Ang Ⅱ -infused Apoe−/− mice via suppression of inflammation, proteolysis, and fibrosis in the aortic wall.**

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Key words: Abdominal aortic aneurysm, Incretin, DPP-4 inhibitor, Angiotensin Ⅱ

Introduction

Abdominal aortic aneurysm (AAA) defined as a permanent dilation of the abdominal aorta is a relatively common condition among the elderly population, especially men[1)]. Gradual progression of a developed AAA can continue for years, increasing the possibility of aneurysm rupture, and the mortality from a ruptured aneurysm is considerably high[2)]. Although effective pharmacological treatment of AAA has been demonstrated multiple times in animal studies, there are no therapeutic approaches to prevent AAA progression in humans, leaving the patients with invasive surgical intervention as their only option. Accordingly, there is a great need for efficient strategies aimed at preventing AAA progression.

Although AAA development is driven by complicated interactions of various mechanisms, there are a number of similarities in the pathophysiology of AAA

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and atherosclerosis[3-6]. Local inflammation in the arterial wall has been implicated as a critical factor in both AAA and atherosclerosis. Chronic inflammation triggers a cascade of events, such as inflammatory cell invasion, increased cytokine, and chemokine release, and abnormal regulation of extracellular matrix (ECM), ultimately resulting in vascular smooth muscle cell (VSMC) apoptosis and disruption of the elastic lamina[7]. We previously reported that administration of native glucagon-like peptide (GLP)-1, native glucose-dependent insulinotropic polypeptide (GIP), or a dipeptidyl peptidase-4 (DPP-4) inhibitor (DPP-4I) significantly suppressed atherosclerotic lesions in the aortic wall of apolipoprotein E-null (Apoe−/−) mice, a representative animal model of atherosclerosis[8-10]. Furthermore, incretin-related agents exert anti-inflammatory effects in vascular endothelial cells, VSMCs, and monocytes/macrophages[9]. These findings suggest that incretins and DPP-4I are potentially effective against AAA as well as atherosclerosis.

Because of several shared risk factors, AAA and atherosclerosis coexist in a substantial number of patients, suggesting the importance of evaluation of AAA in the presence of atherosclerosis. Angiotensin Ⅱ (Ang Ⅱ) is a common agent known to induce AAA in mice by promoting vascular inflammation, ECM degradation, luminal expansion, and thrombosis similar to those observed in humans[11, 12]. In this study, we used male Apoe−/− mice to determine whether native incretins and DPP-4I can suppress Ang Ⅱ-induced AAA and atherosclerosis. Furthermore, we also investigated whether the effect of DPP-4I on the vasculature is dependent on enhanced action of incretins.

Methods

Animal Model

All procedures were approved by the animal care committee of Showa University School of Medicine (approval number, 03112). Apoe−/− mice at 9 weeks of age were purchased from Sankyo Laboratory (Tokyo, Japan). The mice were maintained on an atherogenic diet containing 30% fat, 20% sucrose, 0.15% cholesterol, and 8% NaCl (Oriental Yeast, Tokyo, Japan)[13] throughout the experimental period to exacerbate atherosclerosis. After 4 weeks, the mice were randomly assigned to 5 treatment groups: saline (control), Ang Ⅱ, Ang Ⅱ + GLP-1, Ang Ⅱ + GIP, and Ang Ⅱ + DPP-4I (MK0626; gifted by Merck Sharp & Dohme Corp., NJ, USA). All mice underwent subcutaneous implantation of two osmotic pumps (Alzet model 1002; Durect, CA, USA) under general anesthesia by isoflurane: one for the administration of saline or Ang Ⅱ (2000 ng/kg/min, Wako, Osaka, Japan) and one for saline, GLP-1 [2.16 nmol/kg/day; human GLP-1 (7-36) amide, AnaSpec, CA, USA], or GIP (25 nmol/kg/day; human GIP, AnaSpec)[8]. MK0626 was delivered with food at an approximate dose of 6 mg/kg/day[14, 15]. Some of the mice in the Ang Ⅱ + DPP-4I group were co-administered (Pro3) GIP (25 nmol/kg/day, Abgent, CA, USA), a GIP receptor antagonist, and exendin (9-39) (22 nmol/kg/day, AnaSpec), a GLP-1 receptor antagonist, to examine whether the effect of DPP-4I is dependent on incretin action[8]. The osmotic pumps used in this study provided a reliable rate of release for 2 weeks. Therefore, all the pumps were replaced every 2 weeks.

After 4 weeks of treatment, the mice were fasted for 6 h and sacrificed under general anesthesia by isoflurane. Blood samples were collected from the inferior vena cava and transferred into tubes containing 10 μL of DPP-4I (EMD Millipore, MA, USA) per 1 mL of blood to inhibit inactivation of incretin. The aorta was perfusion fixed with paraformaldehyde followed by phosphate-buffered saline via the left ventricle at a physiologic pressure (100-120 mmHg). The region of the aorta from the root to the bifurcation of the femoral arteries was carefully isolated and excised for the analysis of AAA and atherosclerotic plaque formation.

Metabolic Parameters

Hemoglobin A1c (HbA1c) levels were measured through a cut in the tail vein using A1CNow (Bayer Inc., Leverkusen, Germany) before sacrificing the animals. Plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured using the enzymatic colorimetric method (Wako). Plasma levels of insulin, total GLP-1, active GLP-1, and total GIP were determined using enzyme-linked immunosorbent assay (ELISA) (Ultra-sensitive mouse insulin ELISA kit from Morinaga, Kanagawa, Japan; Multi Species GLP-1 Total ELISA; GLP-1 (active) ELISA; and Rat/Mouse GIP (total) ELISA from EMD Millipore). A small subset of saline, Ang Ⅱ, and Ang Ⅱ + DPP-4I blood samples were collected without DPP-4I to measure DPP-4 activity (DPP-4 Activity Fluorometric Assay Kit, BioVision, CA, USA).

Blood Pressure Measurements

Blood pressure and pulse rate were measured on the day of sacrifice in the fasting state using the tail-cuff method (Model MK-2000ST, Muromachi Kikai, Tokyo, Japan). The average of blood pressure levels taken from consecutive 3-5 times measurement was

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used as a value of individual mouse.

AAA Analysis

To obtain the ratio of the maximum external width of the abdominal aorta to that at the level of the renal branches, the harvested aorta was stained with oil red O and photographed using a dissection microscope. AAA was defined as a dilated aorta with the above ratio >1.5[16, 17]. This ratio was also used for the assessment of AAA progression.

Atherosclerotic Plaque Analysis

Following the assessment of AAA, the aorta was longitudinally dissected and photographed to evaluate atherosclerotic plaque. Atherosclerotic plaque burden was evaluated in the oil red O-stained areas of the lumen surface between the aortic root and thoracic aorta, excluding the regions with AAA. Cases with aneurysm of the thoracic aorta were also excluded from the analysis because the strong staining of the aneurysm with oil red O could affect the results of atherosclerotic plaque assessment. All the measurements were conducted by an investigator blinded to the treatment modality using an image analyzer (ImageJ software; National Institutes of Health, Bethesda, MD, USA)[8].

Histological Analysis

After the analysis of atherosclerotic plaque, the dissected aorta was soaked in 100% ethanol for 48 h to remove oil red O and embedded in a paraffin block to obtain cross sections of AAAs. The cross sections were stained with Elastica van Gieson (EVG) and Masson trichrome stains to evaluate elastin lamina and fibrosis, respectively. Degeneration of elastic laminar was graded as previously described[18]: grade 1, only the external elastic lamella (EEL) is disrupted; grade 2, EEL and the outer middle elastic layer are broken or disrupted; grade 3, EEL and the outer/middle elastic layers experience breakage and/or degradation; grade 4, all elastic layers exhibit signs of breakage and/or degradation. In Masson trichrome-stained sections, blue and purple areas were considered to correspond to fibrotic and thrombotic regions, respectively. The measurements were conducted by a blinded investigator with the ImageJ software.

Real-Time RT-PCR

In separate groups of Apoe−/− mice treated with Ang Ⅱ or Ang Ⅱ+DPP-4I, the thoracic aortas that did not develop aneurysm were harvested, snap frozen in liquid nitrogen, and stored at −80℃ for the analysis of expression of genes related to AAA development or progression, such as matrix metalloproteinases (MMPs and AAA-provoking factors), tissue inhibitors of metalloproteinases (TIMPs and suppressive factors), and inflammatory cytokines and chemokines. Total RNA was extracted from the thoracic aortas using Isogen (NIPPON-GENE, Tokyo, Japan) by following the manufacturer's instructions and used for cDNA synthesized with the ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Gene expression was assessed by real-time RT-PCR using the TaqMan gene expression assay and sequence detection system (ABI PRISM 7900; Life Technologies, CA, USA). The following pre-designed TaqMan probe sets (Life Technologies) were used to detect the expression of the following target genes: *Mmp-2*, Mm00439498_m1; *Mmp-9*, Mm00442991_m1; *Timp-1*, Mm00441818_m1; *Timp-2*, Mm00441825_m1; *Timp-3*, Mm00441826_m1; interleukin-1β (*Il-1β*), Mm00434228_m1; *Il-6*, Mm00446190_m1; tumor necrosis factor (*Tnf*)-α, Mm00443258_m1; monocyte chemotactic protein (*Mcp*)-1, Mm00441242_m1; and transforming growth factor (*Tgf*)-β*1*, Mmp9Mm01178820_m1. 18S ribosomal RNA (Mm03928990_g1) was used as an internal control.

Statistical Analysis

Values are expressed as mean±SD (standard deviation). Occurrence rate between the groups was tested by chi-square test with Bonferroni correction. Analysis of variance (ANOVA) followed by the Tukey's test was used for the comparison of more than two groups, and the unpaired t-test was used for the comparison of two groups. Statistical calculations were performed using the JMP software (version 11; SAS Institute Inc., NC, USA). The significance level was defined as $p<0.05$.

Results

Metabolic Parameters

The metabolic parameters are shown in **Table 1**. There were no differences in the final body weights and daily intake of food and water among the five groups. Although the systolic blood pressure (SBP) was not elevated in the Ang Ⅱ group with statistical significance, the ratio of heart weight to body weight was significantly increased compared with the controls [Ang Ⅱ ($n=28$), 1.27±0.35; saline ($n=11$), 0.70±0.12; $p<0.01$], indicating that the biological activity of Ang Ⅱ given to the mice retained. The Ang Ⅱ+GIP group showed a higher pulse rate than the control and Ang Ⅱ+DPP-4I groups. The HbA1c levels were increased by the Ang Ⅱ+GIP treatment compared

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Table 1. Metabolic parameters of each treatment group

	Saline	Ang Ⅱ	Ang Ⅱ + GLP-1	Ang Ⅱ + GIP	Ang Ⅱ + MK
Number	11	28	13	12	35
Food intake (g/day)	3.6 ± 1.0	3.5 ± 1.1	3.6 ± 0.7	3.6 ± 0.3	3.7 ± 1.2
Water intake (ml/day)	3.8 ± 1.0	3.6 ± 1.1	3.8 ± 0.4	3.8 ± 1.0	3.7 ± 1.2
Final body weight (g)	31.0 ± 1.0	29.5 ± 3.2	29.2 ± 2.9	29.0 ± 2.4	31.0 ± 5.3
SBP (mmHg)	104 ± 20	118 ± 15.9	102 ± 11	108 ± 14	107 ± 18
Pulse rate (beats/min)	556 ± 109	620 ± 90	615 ± 144	671 ± 62*	571 ± 95‡
Insulin (ng/mL)	0.77 ± 0.90	0.64 ± 0.27	0.59 ± 0.14	0.65 ± 0.24	0.58 ± 0.08
HbA1c (%)	4.1 ± 0.3	4.2 ± 0.5	4.3 ± 0.7	5.0 ± 1.0†	4.2 ± 0.6‡
TC (mg/dL)	361 ± 70	339 ± 164	332 ± 123	349 ± 145	402 ± 201
HDL-C (mg/dL)	19 ± 3	15 ± 11	13 ± 7	18 ± 10	17 ± 12
Triglycerides (mg/dL)	60 ± 30	40 ± 21	40 ± 25	45 ± 42	60 ± 53
Total GLP-1 (pmol/L)	39 ± 9	55 ± 38	120 ± 87*†‡	65 ± 53	42 ± 43
Active GLP-1 (pmol/L)	3.0 ± 0.9	3.4 ± 1.6	4.1 ± 1.8	2.8 ± 1.3	5.7 ± 2.2*†‡
Total GIP (pmol/L)	22.3 ± 6.0	20.6 ± 11.1	23.7 ± 8.8‡	34.3 ± 10.6†	29.9 ± 6.4

The values show mean ± SD. One-way ANOVA followed by Tukey's test: *, $p<0.05$ vs. saline; †, $p<0.05$ vs. Ang Ⅱ; ‡, $p<0.05$ vs. Ang Ⅱ + GIP. The number of animal for total GLP-1, active GLP-1, and total GIP levels is 8 to 20 per group. Ang Ⅱ, angiotensin Ⅱ; GLP: glucagon-like peptide; GIP: glucose-dependent insulinotropic polypeptide; MK, MK0626; SBP, systolic blood pressure; HbA1c, hemoglobin A1c; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol.

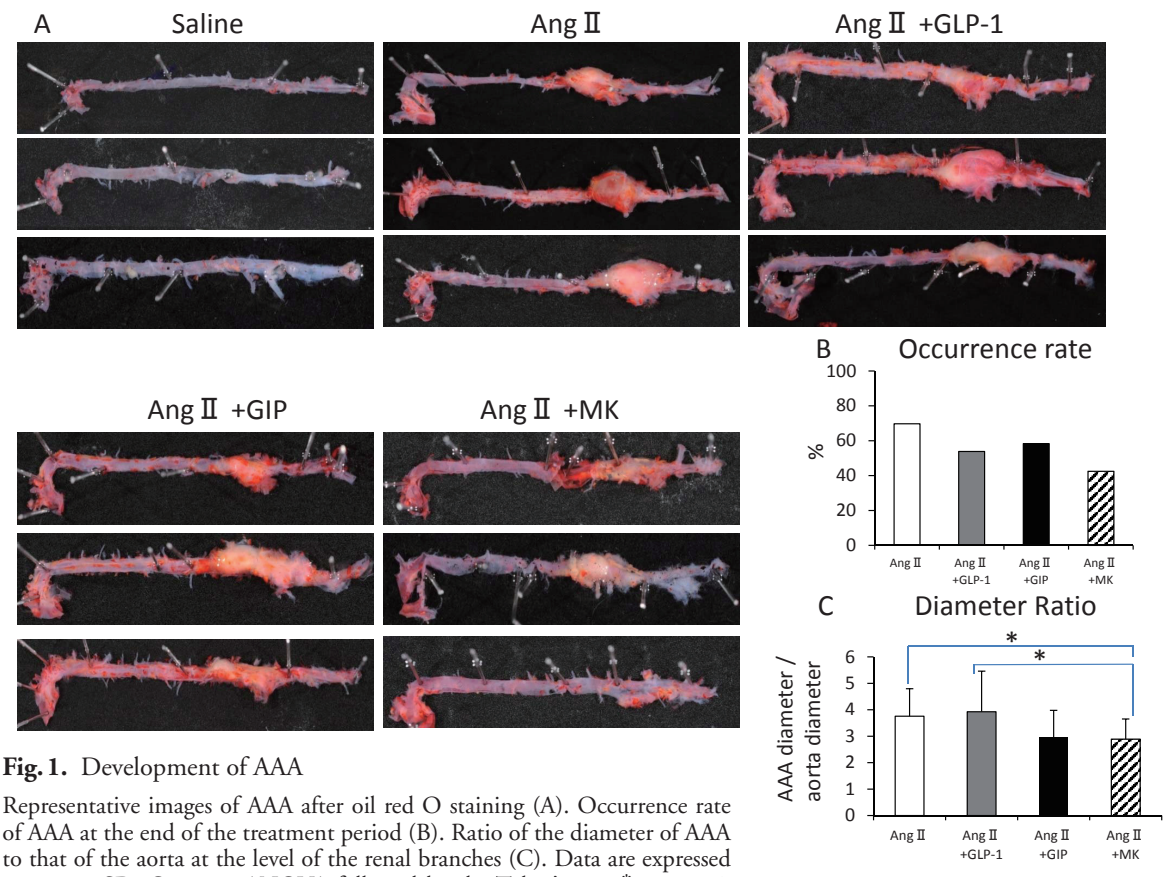

Fig. 1. Development of AAA

Representative images of AAA after oil red O staining (A). Occurrence rate of AAA at the end of the treatment period (B). Ratio of the diameter of AAA to that of the aorta at the level of the renal branches (C). Data are expressed as mean ± SD. One-way ANOVA followed by the Tukey's test: *, $p<0.05$. Ang Ⅱ, angiotensin Ⅱ; GLP: Glucagon-like peptide; GIP: Glucose-dependent insulinotropic polypeptide; MK, MK0626. B: Ang Ⅱ, $n=33$; Ang Ⅱ + GLP-1, $n=13$; Ang Ⅱ + GIP, $n=12$; Ang Ⅱ + MK, $n=40$. C: Ang Ⅱ, $n=23$; Ang Ⅱ + GLP-1, $n=7$; Ang Ⅱ + GIP, $n=7$; Ang Ⅱ + MK, $n=18$.

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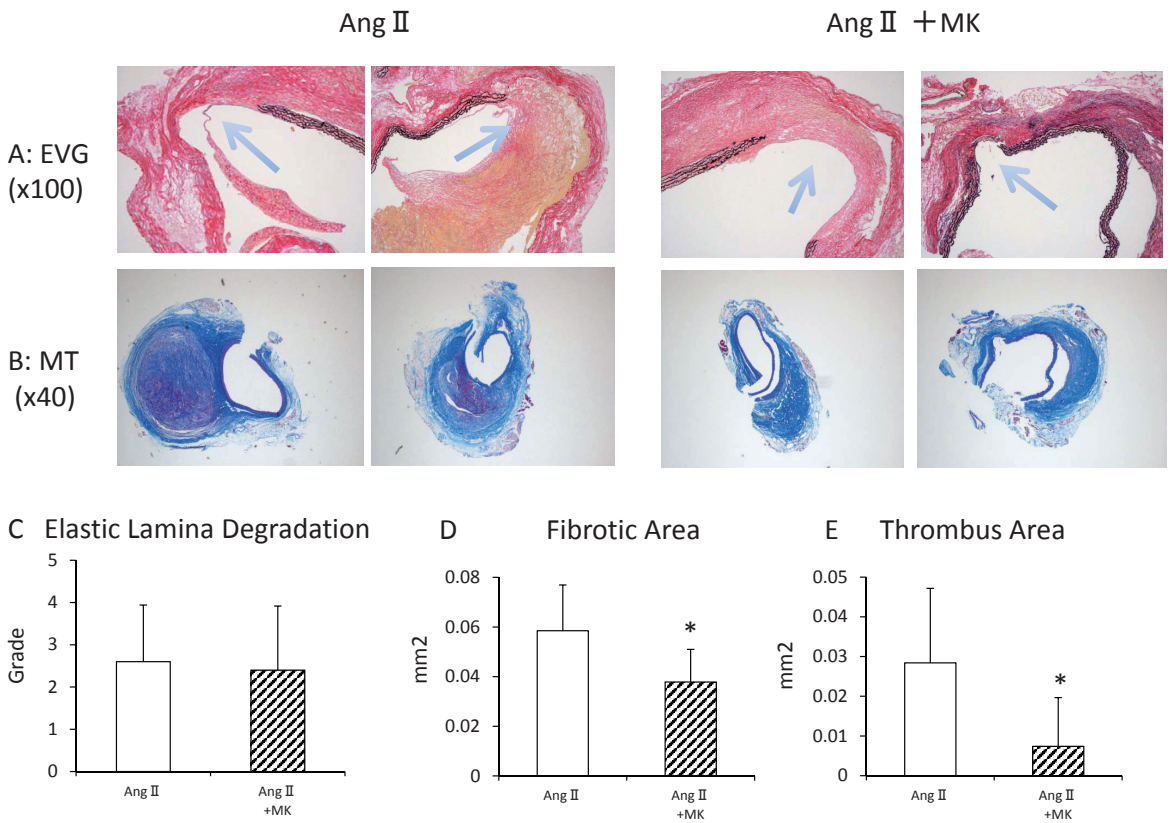

Fig. 2. Elastic laminar degeneration and fibrosis of AAA

Representative images of AAA after Elastica van Gieson (A) and Masson trichrome staining (B). Averages of the elastin degeradation grade (C), fibrotic area surrounding AAA (D), and intra-wall thrombus area (E). The white arrows indicate disrupted elastic layers. The data are expressed as mean ± SD. Unpaired *t*-test: *, $p<0.05$ vs. Ang Ⅱ. $n=6$ per group.

with that by the Ang Ⅱ treatment, while there were no changes in plasma levels of insulin, total cholesterol, HDL cholesterol, and triglycerides among the groups. As expected, plasma levels of total GLP-1 and total GIP increased following the GLP-1 and GIP administration, respectively. DPP-4I supplementation significantly elevated the plasma levels of active GLP-1 without affecting the total GLP-1 levels and tended to increase the total GIP levels ($p=0.15$). To confirm the inhibition of DPP-4 by DPP-4I, we measured plasma DPP-4 activity in small subsets of animals from the study groups. Administration of DPP-4I suppressed >90% of DPP-4 activity compared with the control and Ang Ⅱ treatments (DPP-4 activity: saline, 579 and 586 μU/mL; Ang Ⅱ, 599 and 531 μU/mL; Ang Ⅱ + DPP-4I, 23, 50, 59, and 2 μU/mL).

Development and Progression of AAA

Representative images of AAA from each treatment group are shown in **Fig. 1A**. No AAA was observed in the control animals. AAA occurrence in the Ang Ⅱ group was 70%, whereas that in the DPP-4I group was 40% (**Fig. 1B**). However, this change did not reach statistical significance ($p=0.14$). DPP-4I significantly suppressed AAA progression defined as described in the methods by 23% (**Fig. 1C**) (Ang Ⅱ, 3.7 ± 1.0; Ang Ⅱ + DPP-4I, 2.8 ± 0.8; $p<0.05$). In contrast, GLP-1 and GIP did not show suppressive effects on AAA development and progression.

Elastic Laminar Degradation and Fibrosis of AAA

We stained cross sections of AAA with EVG and Masson trichrome to evaluate elastin layers and perivascular fibrotic and intra-wall thrombus formation, respectively (**Fig. 2A** and **B**). Although the degeneration of elastic laminar did not change (**Fig. 2C**), both fibrotic and thrombus areas were significantly reduced by DPP-4I administration as shown in **Fig. 2D** and **2E** (Fibrosis: Ang Ⅱ, 0.058 ± 0.018 mm^2; Ang Ⅱ + DPP-4I, 0.038 ± 0.013 mm^2; $p<0.05$) (Thrombus area: Ang Ⅱ, 0.028 ± 0.019 mm^2; Ang Ⅱ + DPP-4I, 0.007 ± 0.012 mm^2; $p<0.05$). The degree of fibrosis surrounding an AAA tended to correlate with intra-wall thrombus formation ($r=0.50$, $p=0.06$ according

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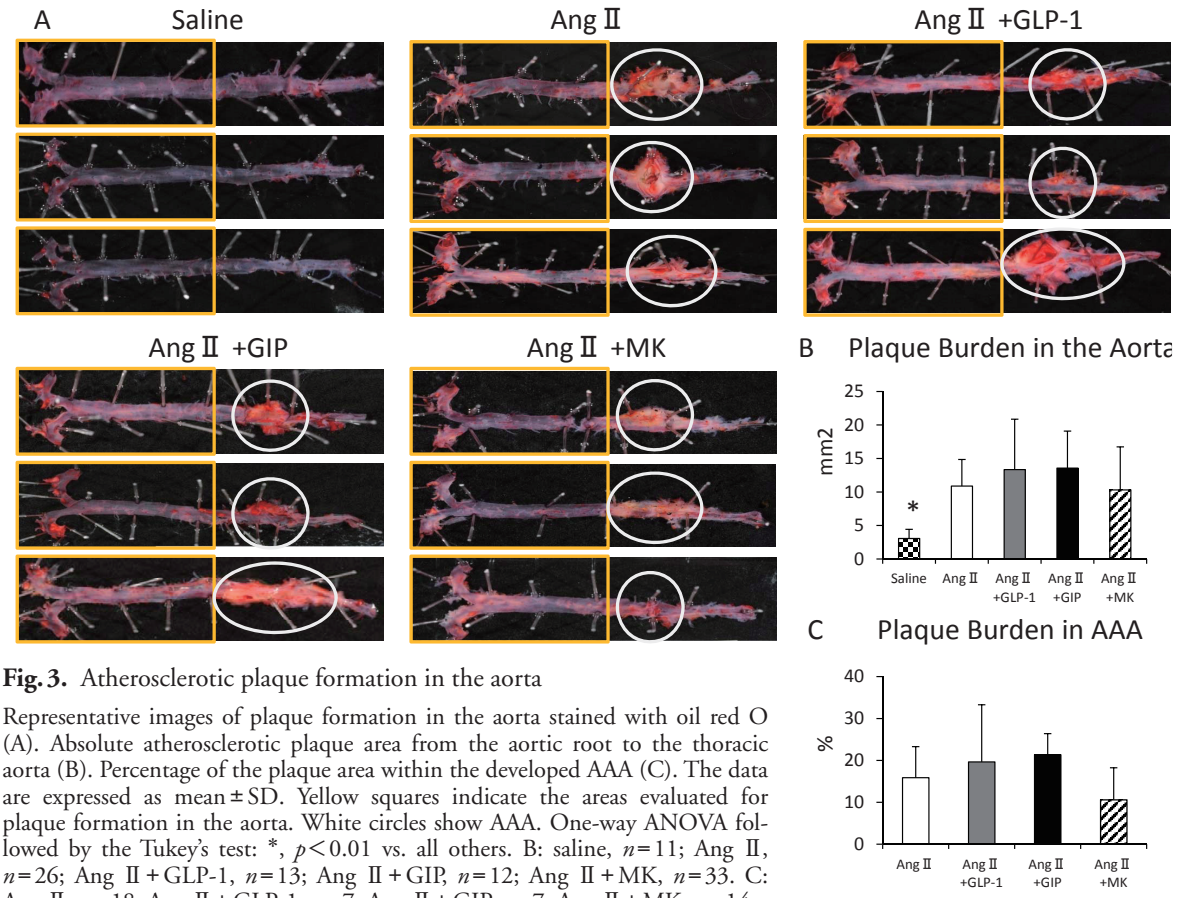

Fig. 3. Atherosclerotic plaque formation in the aorta

Representative images of plaque formation in the aorta stained with oil red O (A). Absolute atherosclerotic plaque area from the aortic root to the thoracic aorta (B). Percentage of the plaque area within the developed AAA (C). The data are expressed as mean ± SD. Yellow squares indicate the areas evaluated for plaque formation in the aorta. White circles show AAA. One-way ANOVA followed by the Tukey's test: *, $p<0.01$ vs. all others. B: saline, $n=11$; Ang Ⅱ, $n=26$; Ang Ⅱ + GLP-1, $n=13$; Ang Ⅱ + GIP, $n=12$; Ang Ⅱ + MK, $n=33$. C: Ang Ⅱ, $n=18$; Ang Ⅱ + GLP-1, $n=7$; Ang Ⅱ + GIP, $n=7$; Ang Ⅱ + MK, $n=14$.

to the Pearson's test).

Atherosclerotic Plaque Formation

Atherosclerotic plaque burden was assessed based on the cumulative oil red O staining-positive area on the surface of the luminal side of the aorta. Representative images are shown in **Fig. 3A**. Ang Ⅱ administration significantly exacerbated the plaque burden compared with saline administration. However, the increased plaque burden formation induced by Ang Ⅱ was not suppressed by GLP-1, GIP, or DPP-4I (**Fig. 3B**). To examine the relation between AAA and atherosclerotic plaque, we also evaluated plaque within the developed AAA. DPP-4I tended to reduce the plaque area inside AAA ($p<0.05$ vs. Ang Ⅱ with unpaired-*t* test).

Effects of Incretin Receptor Antagonists

To determine whether increased incretin activity plays a key role in the mechanism of suppressive effects of DPP-4I on AAA, incretin receptor antagonists were co-administered to some of the mice receiving the Ang Ⅱ + DPP-4I treatment. SBP was lower and TG was higher in the mice treated with DPP-4I and incretin receptor antagonists than in those treated with DPP-4I alone, while the other parameters were comparable between the 2 groups (**Table 2**). Co-administration of incretin receptor antagonists did not reverse the reduction in the incident rate and progression of AAA and fibrosis induced by DPP-4I (**Fig. 4A**, **B**, **C**, and **D**). Furthermore, no change in the atherosclerotic plaque burden in the aorta was observed between the two groups of DPP-4I alone and DPP-4I + incretin receptor antagonists (**Fig. 4E** and **F**).

Effects of DPP-4I on Gene Expression

To obtain further insight into molecular changes related to AAA development and progression, we analyzed the expression of selected genes in the thoracic aortas that did not develop AAA at the time of sacrifice. Among the *Mmp* and *Timp* genes, DPP-4I significantly stimulated *Timp-2* expression and tended to reduce *Mmp-9* expression ($p=0.09$), whereas *Mmp-2*, *Timp-1*, and *Timp-3* expressions were not changed

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Table 2. Metabolic parameters of mice treated with DPP-4I with or without incretin receptor antagonists

	Ang Ⅱ+MK	Ang Ⅱ + MK + Pro3 + EX9
Number	20	15
Food intake (g/day)	3.7 ± 0.9	3.5 ± 0.8
Water intake (mL/day)	3.7 ± 0.9	3.6 ± 0.8
Final body weight (g)	31.0 ± 4.0	31 ± 3.5
SBP (mmHg)	118 ± 22	99 ± 23*
Pulse rate (beats/min)	597 ± 112	522 ± 108
HbA1c (%)	4.2 ± 0.4	4.2 ± 0.4
TC (mg/dL)	414 ± 206	377 ± 201
HDL-C (mg/dL)	16 ± 13	18 ± 8
Triglycerides (mg/dL)	45 ± 27	89 ± 58*

The values show mean ± SD. Unpaired *t*-test: *, $p < 0.05$ vs. Ang Ⅱ + MK. Pro3, (Pro3)GIP; EX9, exendine(9-39).

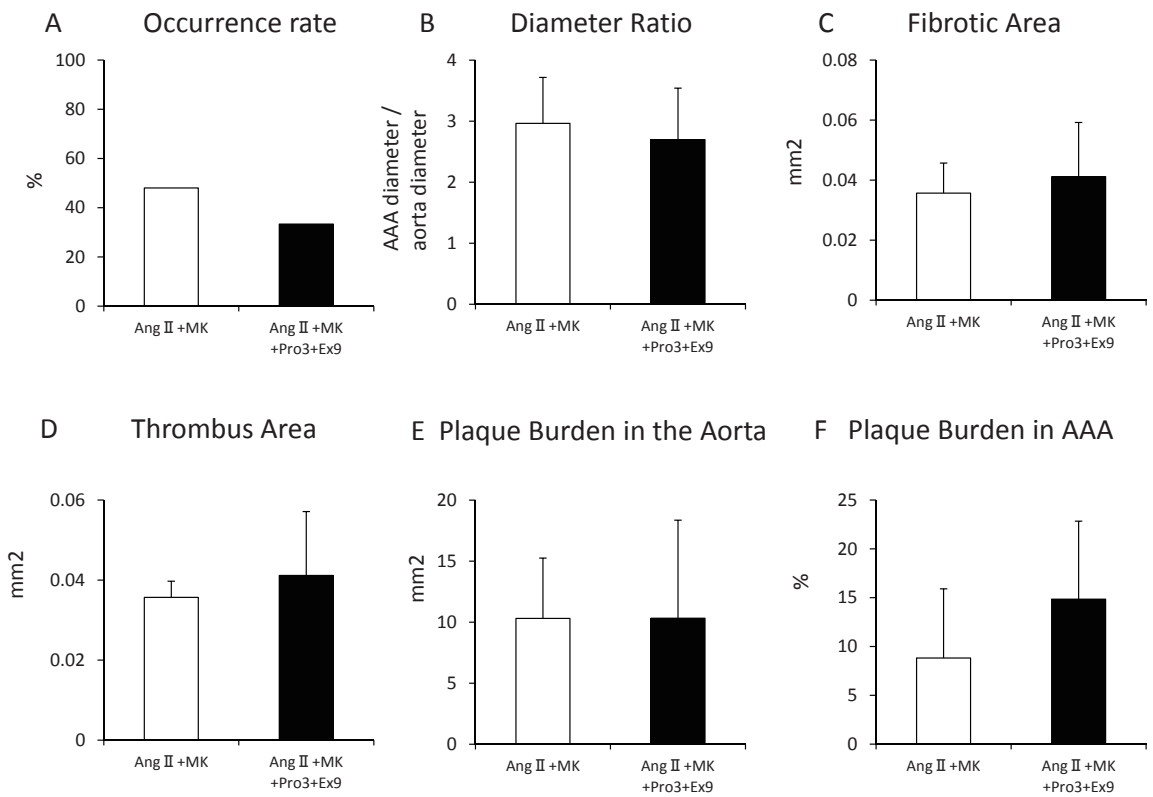

Fig. 4. Effects of the combination of dipeptidyl peptidase-4 inhibitor and incretin receptor antagonists on AAA and atherosclerosis

AAA occurrence rate (A) and ratio of the diameter of AAA to that of the aorta at the level of the renal branches (B). Fibrotic area surrounding AAA (C) and thrombus area within the fibrotic area (D). Atherosclerotic plaque burden in the aorta (E) and AAA (F). The data are expressed as mean ± SD. A: MK, $n = 25$; MK + Pro3 + EX9, $n = 15$. B: MK, $n = 13$; MK + Pro3 + EX9, $n = 5$. C and D: MK, $n = 6$; MK + Pro3 + EX9, $n = 4$. E: MK, $n = 18$; MK + Pro3 + EX9, $n = 15$. F: MK, $n = 12$; MK + Pro3 + EX9, $n = 5$. Pro3, (Pro3) GIP; EX9, exendin (9-39).

(**Fig. 5**). In addition, the *Timp-2*/*Mmp-9* ratio was significantly increased by DPP-4I (**Fig. 5**). The levels of all the inflammatory cytokines and chemokines were markedly elevated in the mouse aortas from the Ang Ⅱ group compared with those from the saline-treated littermate controls (data not shown). DPP-4I admin-

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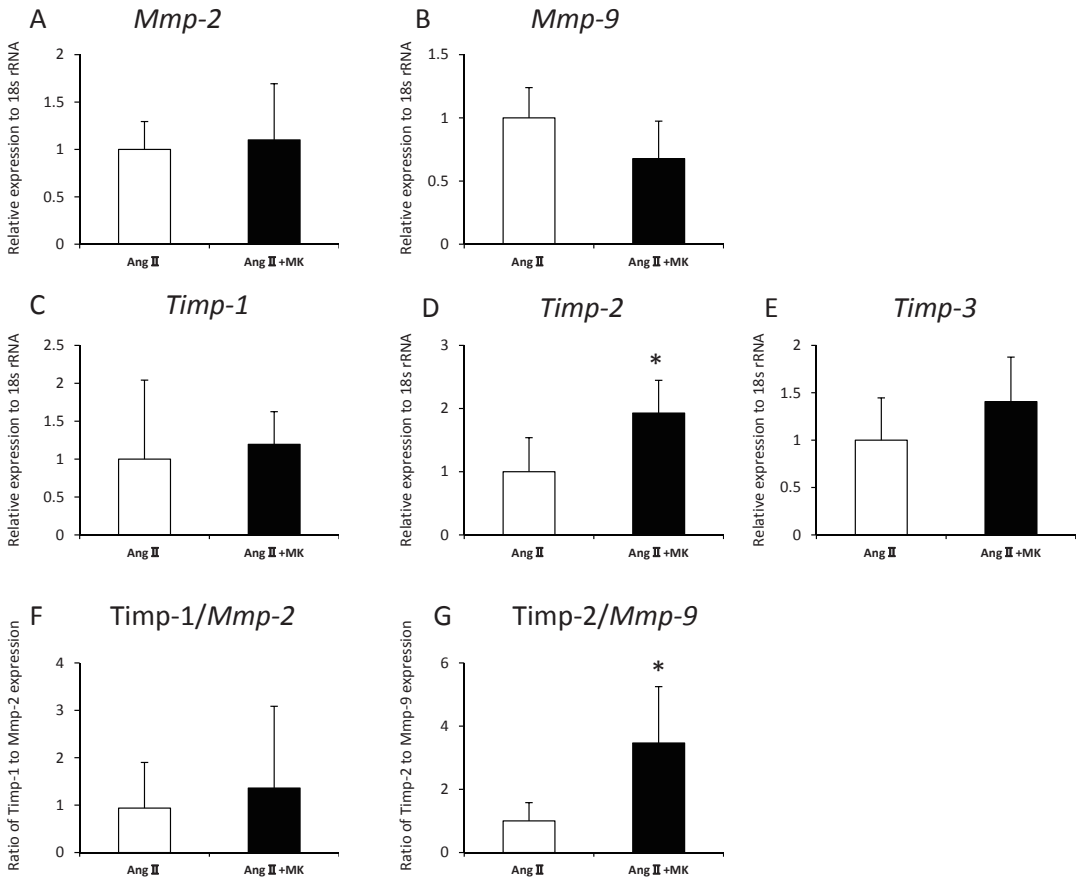

Fig. 5. Effects of DPP-4I on the expression of MMPs and TIMPs

Mmp-2 (A), *Mmp-9* (B), *Timp-1* (C), *Timp-2* (D), and *Timp-3* (E). *Timp-1*/*Mmp-2* ratio (F) and *Timp-2*/*Mmp-9* ratio (G). The data are expressed as mean ± SD. Unpaired *t*-test: *, $p < 0.05$ vs. Ang Ⅱ. $n = 4$-7 per group.

istration significantly suppressed *Il-1β* expression. In contrast, *Il-6*, *Tnf-α*, and *Mcp-1* expressions were not affected by DPP-4I (**Fig. 6**).

Discussion

This is the first study to demonstrate that a DPP-4I, MK0626, can prevent Ang Ⅱ-induced AAA progression in the presence of atherosclerosis in a mouse model, in part, in an incretin-independent manner. To obtain a higher occurrence rate of AAA, we used an infusion dose of Ang Ⅱ (2000 ng/kg/min) that was twice the usual dose[11, 12]. Despite the high Ang Ⅱ dose, DPP-4I successfully suppressed AAA progression. DPP-4I tended to reduce the occurrence of AAA; however, this reduction did not reach statistical significance. There are two papers reporting effects of DPP-4I on AAA[19, 20]. In accordance with our results, alogliptin administration significantly suppressed the progression of AAA. Lu HY *et al.* reported that sitagliptin suppressed AAA occurrence in a dose dependent manner in Apoe−/− mice infused Ang Ⅱ at the dose of 1000ng/kg/min. It is possible that the difference in protocol, such as age, dose of Ang Ⅱ, and type of diet, may explain the failure in the suppression of AAA occurrence by DPP-4I in our study. In addition, both the native incretins and DPP-4I failed to suppress the severe atherosclerosis induced by Ang Ⅱ, suggesting that the atherogenic effects of Ang Ⅱ at this high dose outweighed the anti-atherogenic action of the incretin-related agents. Although chronic inflammation in the arterial wall is the main pathophysiology of both AAA and atherosclerosis, it appears to be more deeply involved in the formation of aneurysms[6]. Inflammatory cells secrete proteolytic enzymes, such as MMPs, thereby disrupting the integrity of the vessel wall[21]. Therefore, massive invasion of inflammatory cells into the aortic wall is necessary for the development of AAA. In contrast, accumulation of inflammatory cells is usually limited within plaque lesions,

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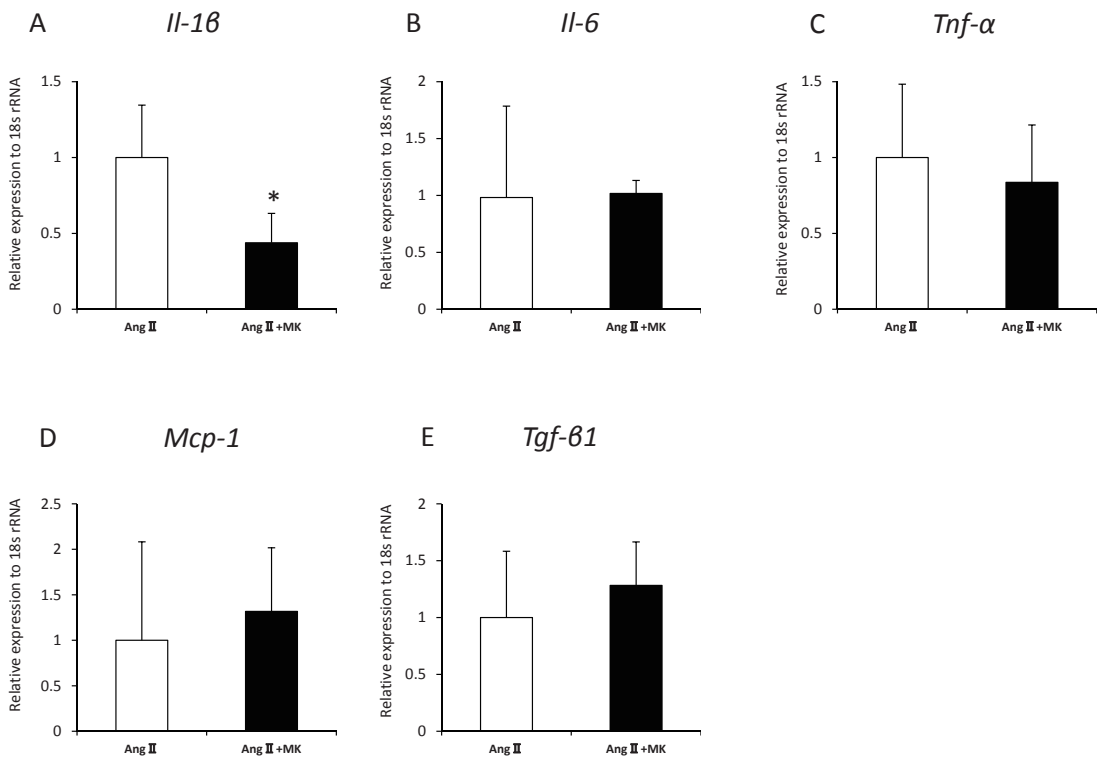

Fig. 6. Effects of DPP-4I on the expression of inflammatory cytokines and chemokines

Il-1β (A), *Il-6* (B), *Tnf-α* (C), *Mcp-1* (D), and *Tgf-β1* (E). The data show expression levels relative to that of 18S ribosomal RNA and are expressed as mean ± SD. Unpaired *t*-test: *, $p < 0.01$ vs. Ang Ⅱ. $n = 4$-7 per group.

and the integrity of the vessel wall is maintained in atherosclerosis. These differences may explain the fact that DPP-4I primarily suppressed AAA progression rather than atherosclerosis in our animal model.

The protective effects of DPP-4I against AAA were not abolished by the co-infusion of (Pro3) GIP and exendin (9-39). Although exendin (9-39) was originally identified as a high potency antagonist of GLP-1 receptor (GLP-1R)[22], several studies showed that this peptide is not a highly selective GLP-1R antagonist[23, 24] and may exert some effect through pathways independent of GLP-1R[25]. To address the effects of (Pro3) GIP or exendin (9-39) on the vasculature, we previously investigated their isolated effects in a mouse model of atherosclerosis using Apoe−/− mice. We confirmed that neither (Pro3) GIP nor exendin (9-34) showed any effects on atherosclerosis or macrophage formation[8]. Given the fact that native incretins did not show beneficial effects on AAA, it could be reasonable to consider that DPP-4I exerted protective effects against AAA, at least in part, independent of incretin actions even though there are limitations in the use of (Pro3) GIP and exendin (9-34) as incretin receptor antagonists. To obtain further insight into the role of incretins in the effects of DPP-4I, MK0626, and those of other DPP-4 inhibitors on AAA, an investigation using both GLP-1 and GIP receptors knockout mice following pharmacological study to compare different DPP-4 inhibitors is required.

We found that the IL-1β expression stimulated by Ang Ⅱ was significantly reduced by DPP-4I in the aorta. IL-1 has been shown to initiate the inflammatory cascade and work as the gatekeeper of inflammation[26]. It has been reported that the IL-1β gene and protein expression levels were substantially elevated in human AAAs[27, 28], and circulating IL-1β levels were elevated in patients with AAA[29]. Moreover, genetic depletion of IL-1β and the IL-1 receptor as well as pharmacological inhibition of the IL-1β receptor suppressed AAA formation in mice[30]. Ta *et al.* reported that a DPP-4I suppressed IL-1β expression in cultured human monocytes[31]. Collectively, these results indicate that decreased IL-1β expression can in part explain the protective effect of DPP-4I against AAA formation.

In contrast to IL-1β, the increased expressions of IL-6, MCP-1, and TNF-α with the Ang Ⅱ treatment were not affected by DPP-4I. IL-6 and TNF-α levels have been shown to be elevated along with the IL-1β

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level in the serum and aneurysms in patients with ruptured AAA[29, 32]. However, their exact roles in the development and progression of AAA are not fully understood. Pharmacological intervention of TNF-α attenuated the formation of calcium chloride-induced AAA, whereas the genetic deletion of the TNF-α receptor failed to improve Ang Ⅱ-induced AAA. Surprisingly, inhibition of MCP-1 activity via overexpression of a dominant-negative form of MCP-1 accelerated AAA progression, whereas selective inhibition in leukocytes using siRNA delayed it. Further studies are required to clarify the general mechanism of inflammation involvement in AAA.

MMPs are deeply involved in the degradation of ECM, and inadequate activation of MMPs leads to elastin degradation in the aortic medial wall, causing AAA formation[33]. Tissue inhibitors of TIMPs are the major endogenous regulators of MMP activity in the tissue and are regulated at the transcriptional level by various cytokines and growth factors. The following four isoforms of TIMP are determined: TIMP-1,-2,-3, and -4. Although these TIMPs inhibit activity of MMPs, the efficacy of MMP inhibition varies with each TIMP[34]. A balance in tissue between MMPs and TIMPs controls ECM deposition and degradation, which is related to the pathogenesis of various diseases including vascular disease. Earlier report suggested that the overexpression of TIMP-2 specifically reduced atherosclerotic plaque lesions and inhibited migration and apoptosis of macrophages and foam cells, whereas that of TIMP-1 did not[35], but the role and regulation of TIMP-2 in AAA formation remains unknown. There is one report that TIMP-2 expression is not altered by IL-1β in human chondrocytes[36], whereas TIMP-1 expression is significantly elevated. This result suggests that mechanisms other than decreased IL-1β are involved in increased TIMP-2 expression by DPP-4I; however, it should be noted whether the result from chondrocytes can be applicable to cells involved in AAA formation. Further investigations are necessary to obtain better understanding about how DPP-4I upregulates TIMP-2 expression in AAA.

Ang Ⅱ has been shown to promote the synthesis of MMP-9 in the aorta[37]. Recent studies demonstrated that a DPP-4I suppressed AAA dilatation in experimental AAA models, which was accompanied by decreased expression and activity of MMP-9 in the aortic aneurysms[19, 20]. As mentioned above, genetic deletion of IL-1β suppressed AAA formation[30], and this suppression was accompanied by decreased macrophage infiltration and MMP-9 activity in AAA, whereas MMP-2 activity was not changed, indicating that IL-1β are involved in the regulation of MMP-9 activity. It has also been reported that a DPP-4I inhibited toll-like receptor 4-mediated extracellular signal-regulated kinase (ERK) activation and ERK-dependent MMP expression in monocytes[31, 38]. ERK activation is known to be involved in Ang Ⅱ-induced AAA formation[39]. Therefore, a DPP-4I may suppress MMP-9 expression through IL-1 β expression and ERK activation in AAA formation. In contrast to MMP-9, the findings in MMP-2 expression and activity in AAA is controversial. Two animal studies reported a consistent finding that DPP-4I suppressed MMP-2 expression in addition to MMP-9 expression[19, 20]. However, we demonstrated significantly decreased IL-1β and increased TIMP-2 expressions, but the change in MMP-2 or MMP-9 expression was not detected. The dissociation between this study and previous studies about MMP-2 and MMP-9 expression would be because of the differences in the condition of the collected aortic samples. We used non-dilated thoracic aortas because little is known about the molecular changes by DPP-4I in non-dilated aortas, which represent the earliest stage of AAA formation. In one of the previous studies evaluating an effect of DPP-4I on AAA, molecular changes were assessed with dilated abdominal aortas with more than 1.5-fold expansion compared with non-dilated part of the abdominal aorta, which was defined as aortic aneurysm. An aortic aneurysm has heterogeneous characteristic: infiltration of macrophages into the aortic wall occurs in the non-dilated area, apoptosis of medial VSMCs and disruption of elastic lamia are in progress in the marginal area, and accumulation of extracellular matrix develops in the dilated area with disappeared VSMCs and elastic lamina in the wall[40]. It is reported that the expression of MMPs is different in the segments of AAA: MMPs show higher expression in the smooth muscle cells at the borders of the areas of medial degeneration than in other regions[41]. Because it is difficult to distinguish them, there is a limitation in understanding where observed findings represent in an experiment using the whole specimen of obviously formed aortic aneurysms (developed aortic aneurysm). The use of non-dilated aortas allows evaluating earlier changes in molecules in aortic aneurysm formation at a stage with elastic lamina preserved. In the other study[19], the aortas with elastase-perfusion showed decreased MMP-2 and -9 activity 7 days after the perfusion, while both activities were increased at a later time point. However, the initiating event in aortic aneurysm formation by elastase perfusion is the degradation of elastic lamina of the aortic wall, which is different from Ang Ⅱ-induced aortic aneurysms in which macrophage infiltration into the

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aortic wall occurs prior to a change in the elastic lamina. Our findings indicate that the changes in IL-1β and TIMP-2 expression by DPP-4 inhibitor would be an earlier event than the change in other molecules in the non-dilated aortas in this model. There are some differences in the characteristics between the thoracic and abdominal aortas because of their differing origin, but heterogeneity within these regions has not been well defined[42]. In fact, the thoracic aorta is not a region prone to aortic aneurysm formation compared with the abdominal aorta. Although we confirmed that the thoracic aortas developed aortic aneurysms at a later time point than the end of this study, further investigations are necessary to determine whether the findings in non-dilated thoracic aortas could be applicable for non-dilated abdominal aortas.

In this study, the DPP-4I administration reduced intra-wall thrombus formation within AAA, and this was accompanied with a decrease in the AAA fibrotic area. Previous studies showed that intra-wall thrombus formation that was localized to the dissected medial occurred within 4-10 days of Ang II infusion in Apoe−/− mice. Formed thrombus subsequently leads to collagen deposition, resulting in increased size of AAA in addition to dilated aortic lumen[40]. There is no report showing a direct effect of DPP-4I on the coagulation system. Therefore, we speculate that DPP-4I reduced medial dissection, following intra-wall thrombus formation through suppression of ECM degeneration without affecting the coagulation system. Disruption of elastic laminar, which was not changed by DPP-4I in the present study, plays an important role in the development of medial dissection of AAA in addition to the development of AAA. Furthermore, degeneration of ECM caused by an imbalance between MMPs and TIMPs has been shown to contribute to the development of medial dissection[43-45]. For example, low TIMP-2 to MMP-2 and TIMP-2 to MMP-9 ratios were related to aortic dissection in human AAA samples[45]. It is possible that the increased TIMP-2/MMP-9 ratio by DPP-4I contributed to improve degeneration of ECM caused by MMP-TIMP imbalance, possibly resulting in decreased intra-wall thrombus formation via prevention of medial dissection. As a limitation of this study, we did not evaluate MMP-2 and -9 activities, which exactly reflect the over-all balance between TIMPs and MMPs. Thus, evaluation of MMP activity would provide more insight into the underlying mechanisms.

Although in our previous studies, DPP-4I and incretins reduced atherosclerotic plaque development in Apoe−/− mice[8-10], these effects were not observed in Ang II-infused Apoe−/− mice. In this study, DPP-4I failed to suppress atherogenesis in the aorta but reduced the plaque area in the developed AAA. Local atherosclerotic plaque could promote AAA progression via oxidative stress, which was shown to accelerate Ang II-induced AAA formation[46], in addition to inflammatory cytokines and chemokines. It has been reported that plaque formation is not observed before AAA development[40], suggesting that the observed change in local plaque burden caused by DPP-4I may affect AAA progression rather than development.

DPP-4 is shown to inactivate GLP-1 and GIP by cleaving them at the N-terminus[47]. It is also reported that DPP-4 cleaves multiple peptides, including stromal derived factor (SDF)-1 alpha, which is also known as C-X-C motif chemokine 12 (CXCL12), in addition to incretins[48]. There are reports showing that SDF-1α demonstrated protection against ischemic tissues and injured arteries[49, 50]. In contrast, a recently published study demonstrated that neutralization of SDF-1 by antibody reduced intracranial aneurysm formation thorough suppression of angiogenesis and cell proliferation in the wall of aneurysms[51], indicating that increased SDF-1α by inhibition of DPP-4 may compromise the beneficial effects of DPP-4I on AAA. Interestingly, treatment with DPP-4I did not increase the circulating SDF-1α level in Ang II-infused Apoe−/− mice that were fed an atherogenic diet[20]. We did not evaluate SDF-1α levels because an investigation for potential roles of SDF-1α in AAA was beyond our scope, which aimed to determine incretins' role in AAA formation. However, a change of SDF-1α by DPP-4I is warranted for an investigation to obtain further insight into the mechanisms of DPP-4I's effects on AAA.

Conclusions

This is the first study demonstrating that DPP-4I administration can prevent AAA progression without affecting systemic atherosclerosis via suppressing IL-1β and stimulating TIMP-2 expression. Furthermore, this protective effect of DPP-4I was, in part, independent of incretins. These findings provide evidence that DPP-4I may serve as a potential therapeutic strategy for AAA.

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Authors' Contributions

KK and MH conducted major parts of experiments and equally contributed to the present study as first authors. YM conducted experiments with KK and MH, analyzed data, and drafted the manuscript. MT, HK, KS, MT, and MN helped KK and MH with the animal experiments. TW contributed to the study design, discussion, and revise for the manuscript. TH designed the study, and drafted, reviewed, and finalized the manuscript.

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