Suppression of Abdominal Aortic Aneurysm Formation in Mice by Teneligliptin, a Dipeptidyl Peptidase-4 Inhibitor

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Aim: Dipeptidyl peptidase-4 (DPP-4) inhibitors lower blood glucose levels through inhibition of incretin degradation, which stimulates insulin secretion. Recent studies reported that DPP-4 inhibitors suppressed atherogenesis in apolipoprotein E-knockout (ApoEKO) mice. In this study, we investigated whether teneligliptin, a DPP-4 inhibitor, affects the development of abdominal aortic aneurysms (AAA) in ApoEKO mice.

Methods: ApoEKO mice were fed a high-fat diet (HFD) and infused with angiotensin (Ang) II by osmotic mini pumps for 4 weeks to induce AAA with (DPP-4i group) or without (control group) teneligliptin administered orally from 1 week before HFD and Ang II infusion to the end of the experiment. Confluent rat vascular smooth muscle cells (VSMCs) were serum-starved for 48 hours, then incubated with or without teneligliptin for another 24 hours and stimulated with Ang II.

Results: Treatment with teneligliptin significantly reduced the AAA formation rate (30.7% vs. 71.4% vs. control, P < 0.05), aortic dilatation (1.32 ± 0.09 mm vs. 1.76 ± 0.18 mm in the control, P < 0.05) and severity score (0.75 ± 0.28 vs. 1.91 ± 0.4 in the control, P < 0.05). Elastin degradation grade was also attenuated in DPP-4i group (2.83 ± 0.17 vs. 3.45 ± 0.16 in the control, P < 0.05). The number of macrophages infiltrating into the abdominal aorta was decreased in the DPP-4i group (51.8 ± 29.8/section vs. 219.5 ± 78.5/section in the control, P < 0.05). Teneligliptin attenuated Ang II-induced phosphorylation of extracellular signal-regulated kinase (ERK) and Akt, and mRNA expression of monocyte chemoattractant protein-1 in VSMCs.

Conclusion: Treatment with teneligliptin suppressed AAA formation in ApoEKO mice with HFD and Ang II infusion. Suppression of macrophage infiltration by teneligliptin may be involved in the inhibition of AAA formation.

Key words: Abdominal aortic aneurysm, Dipeptidyl peptidase-4 inhibitor, Angiotensin II, ERK

Introduction

Abdominal aortic aneurysm (AAA) is defined as an increase in the diameter of the abdominal aorta more than 150% of that of a normal aorta. The risk factors for the development of AAA include smoking, hypertension, an elevation of cholesterol level, and pre-existing atherosclerotic vascular disease. AAAs grow gradually; however, their consequences are catastrophic once rupture of the AAA occurs. It is well known that there is a positive correlation between the diameter of AAAs and the incidence of AAA rupture. Although treatment with angiotensin-converting enzyme inhibitors or angiotensin II (Ang II) receptor blockers reduce the
mortality from AAA in humans, there is no established medical therapy that can attenuate AAA growth in humans.

Inflammation and degeneration of the aortic wall are thought to be key mechanisms of aortic dilatation leading to AAA formation. Several drugs were reported to inhibit AAA growth in animal models of AAs, which includes statins, an inhibitor of c-Jun N-terminal kinase, and Ang II receptor blockers. The effects of these reagents mainly depend on the suppression of aortic inflammation. However, none of them is proven to suppress the progression of human AAs.

Dipeptidyl peptidase-4 (DPP-4) is a protease that degrades incretins such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide. Incretins promote insulin secretion from pancreatic beta cells and reduce blood glucose levels. Therefore, inhibition of DPP-4 by DPP-4 inhibitors, a new class of drug for type 2 diabetes, attenuates hyperglycemia through an increase in incretin availability and insulin levels.

In addition to incretins, DPP-4 has various substrates such as protease-activated receptor-2, stromal cell derived factor (SDF)-1α, and neuropeptide Y, indicating that DPP-4 inhibitors may have diverse effects by increasing the protein levels of these molecules. DPP-4 inhibitors improved endothelial function and attenuated atherogenesis in apolipoprotein E-knockout (ApoEKO) mice without changing serum glucose levels. Treatment with a DPP-4 inhibitor improved left ventricular function after myocardial infarction in mice. These studies may suggest beneficial cardiovascular effects of DPP-4 inhibitors possibly through incretin-independent mechanisms.

Recent studies suggest that macrophages play an important role in the initiation and progression of AAA and macrophages are known to express DPP-4. Furthermore, inhibition of DPP-4 inhibits proliferation of vascular smooth muscle cells (VSMC). We, therefore, examined whether teneligliptin, a DPP-4 inhibitor, suppresses AAA formation in a murine model.

Materials and Methods

Materials

Dulbecco’s Modified Eagle Medium (DMEM, 11965-118) was purchased from Gibco-BRL, Invitrogen Co. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from SAFC Biosciences Inc. (Lenexa, KS, USA, 10437-028). Bovine serum albumin (BSA, A9056), an antibody against α-tubulin (T-6074), and 30% polyoxymethylene lauryl ether solution (03-3170) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against phosphorylated (p) extracellular signal-regulated kinase (ERK, 9106) and pAKT (9271), and total ERK (9102) and AKT (9272) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). An antibody against MAC2 (CL942AP), Ang II (4001), Glycyl-L-proline 4-methylcoumaryl-7-amide (Gly-Pro-MCA, 3090-v), and 7-Amino-4-methylcoumaryl (Gly-Pro-MCA, 3090-v) were purchased from Peptide institute Inc. (Osaka, Japan). Osmotic mini pumps were purchased from Alzet (Cupertino, CA, USA, 2004). A high-fat diet (HFD) containing 40% of calories from fat (19.82% casein, 0.3% L-cysteine, 3.7458% corn starch, 1.25% α corn starch, 34% sucrose, 20.0% milk fat, 1.0% soybean oil, 9.98% maltodextrin, 5.0% cellulose, 0.15% cholesterol, 1.0% vitamin mixture, 3.5% salt mix, 0.25% choline bitartrate, 0.0042% tertiary butyl hydroquinone) was purchased from KBT Oriental Co. Ltd. (Saga, Japan). Teneligliptin was supplied by Mitsubishi Tanabe Pharma (Osaka, Japan).

Murine AAA Model

All procedures were approved by the Animal Care and Use Committee, Kyushu University and conducted in accordance with the institutional guidelines. ApoEKO mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Development of AAA was induced as described previously with minor modifications. Briefly, ApoEKO mice (12 week old male mice) were fed a HFD and administered Ang II for 4 weeks via osmotic mini pumps implanted subcutaneously. Osmotic mini pumps were implanted under anesthesia by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg). At 4 weeks after the operation, the systolic blood pressure (SBP) and heart rate (HR) were measured with the tail-cuff method in a conscious state (BP-98A-L, Softron, Tokyo, Japan). Teneligliptin was given orally through drinking water and started 1 week before HFD and Ang II treatment (DPP-4i group). The approximate intake of teneligliptin was 30 mg/kg/day. The control (CTRL) group was given HFD and Ang II. The normal group was fed a normal chow.

Assessment of AAA

Twenty eight days after implantation of osmotic mini pumps, mice were anesthetized with sevoflurane. Luminal diameter of the aorta between the superior mesenteric artery and renal artery bifurcation was determined by using the VEVO2100 Ultrasound system with a 40 MHz MS550D transducer (Visual Sonics, Toronto, Canada). Mice were then euthanized with diethyl ether and the aortic tree, aortic root to the iliac bifurcation, was dissected from the surrounding tissue. The external diameter was determined using ImageJ software. The severity of AAA development was classi-
fied based on Daugherty’s classification\(^\text{18}\). An AAA is defined as a 1.5-fold enlargement of the aortic diameter compared with ApoEKO mice fed a normal chow.

**Histological Analysis**

Paraffin-embedded abdominal aortae were cut into 7 µm sections. Cross-sections of the aorta were subjected to Elastica van Gieson (EVG) staining and Mac-2 immunostaining. Cross-sections were prepared every 600 µm to check the elastic lamina degradation grade of the aorta. The elastic lamina degradation grade was scored by the criteria previously reported (Grade 1: almost normal, Grade 2: mild degree degradation, Grade 3: high degree degradation, Grade 4: severe degree degradation)\(^\text{18}\). For Mac-2 immunohistochemical staining, paraffin-embedded tissue sections were deparaffinized, rehydrated and then autoclaved in 10 nmol/l citrate buffer for antigen retrieval. After quenching of endogenous peroxidase, sections were incubated with an anti-mouse Mac-2 antibody at 4°C overnight. After incubation with a peroxidase-conjugated secondary antibody (Simple Stain MAX-PO; NICHIREI BIOSCIENCES INC. Tokyo, Japan, 414311), sections were incubated in diaminobenzidine solution and counter stained with hematoxylin. The section from the largest diameter of the suprarenal abdominal aorta was selected for macrophage staining. The number of Mac-2 positive cells was quantified by counting the total number of diaminobenzidine positive cells per section.

**Assessment of Serum DPP-4 Activities**

Serum DPP-4 activity was measured by a method reported previously\(^\text{19}\). Briefly, whole blood was collected at sacrifice. The blood was centrifuged at 3000 rounds per minute for 10 minutes. The supernatant was collected as serum. The serum was incubated at 37°C for 15 hours with reaction buffer. Then, gels were stained with 0.5% Coomassie Blue R-250 at room temperature for 60 minutes and destained for 30 minutes, so that protein bands with gelatinolytic activity were easily identified as clear lytic bands. Band intensities were quantified using ImageJ software.

**Western Blotting**

For western blotting, preparation of aortic tissue lysate was performed as described above. Equal amounts (10 µg) of protein samples were subjected to SDS/PAGE and transferred onto PVDF membranes (Immobilon-P; Millipore). After blocking with 5% skimmed milk, the membrane was incubated with primary antibodies, followed by exposure to the secondary antibodies. α-tubulin immunoblotting was used as a protein loading control. Signals were detected using an ECL Prime Western Blotting Detection reagent (GE Healthcare UK Ltd., Buckinghamshire, England, RPN 2232). Chemiluminescence was detected by ImageQuant LAS 4010 (GE healthcare). The signals were quantified by ImageJ software.

**Measurement of Serum Levels of Triglyceride, Cholesterol, and Glucose**

Serum triglyceride and total cholesterol levels were determined by commercially available kits, Triglyceride E-test Wako (Wako, Osaka, Japan 432-40201) and Cholesterol E-test Wako (Wako 439-17501), respectively. Serum glucose levels were determined by Glu-est Évry (SANYA KAGAKU KENKYUSHO Co. Ltd. Aichi, Japan, 86532617).

**qRT-PCR**

RNA isolation kits (Roche Ltd. Basel, Switzerland, 12033674001) were used to extract total RNA from VSMCs according to the manufacturer’s protocol. Total RNA from VSMCs (1 µg) was reverse-transcribed with ReverTra Ace qPCR RT kit (TOYOBO CO., LTD, Osaka, Japan, TRT-101). Real-time qPCR analysis was performed using the Power SYBR Green PCR Master mix (Life Technologies Japan Ltd., Tokyo, Japan, QPS-201) and the Applied Biosystems 7500 Real-Time PCR system according to the manufacturer’s protocol. The following primers were used to determine the expression of monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-6: Expression of hypoxanthine phosphoribosyltransferase (Hprt) was used as an internal control to normalize the expression of IL-6 and MCP-1 because expression of Hprt showed a relatively constant Ct number in our experiments.

Il6, F; 5’-CCACTTCAAAAGTGAGGCTTA-3’,
Teneligliptin Suppressed Degradation of Elastic Laminae

Degradation of medial elastic laminae is the histological feature of AAA. EVG staining revealed that teneligliptin attenuated elastic fiber degradation (2.83±0.17 vs. 3.27±0.16 in the CTRL group, P<0.01). In almost all mice in the CTRL group, the elastic fiber was stretched and digested (Fig. 2A, B). However, degradation of the elastic laminae was hardly seen in the DPP-4i group. Gelatin zymography using protein extracts from AAA tissues revealed that teneligliptin suppressed the activity of matrix metalloproteinase (MMP)-9 but not MMP-2 in the aortic wall (Fig. 2C). However, the decrease in MMP-9 activity was not statistically significant.

Teneligliptin Inhibited Macrophage Migration into the Aortic Wall

The number of macrophages that accumulated...
To explore the mechanism for the suppression of AAA formation by teneligliptin, we examined the effects of teneligliptin on Ang II-induced signaling and inflammatory responses to Ang II in VSMCs.

In the aortic wall, the AAA incidence and severity were lower in the DPP-4i group compared with the CTRL group (Fig. 3). The aortic external diameter was decreased in the DPP-4i group compared with the CTRL group (51.75 ± 29.8/section vs. 219.45 ± 78.5/section in the CTRL group, *P < 0.05) (Fig. 3).

DPP-4 Inhibitor Attenuated Inflammatory Responses to Ang II in VSMCs

To explore the mechanism for the suppression of AAA formation by teneligliptin, we examined the effects of teneligliptin on Ang II-induced signaling and inflammatory responses in VSMCs.
gene expression in VSMCs. Pretreatment with teneligliptin suppressed Ang II-induced ERK and Akt phosphorylation (Fig. 4A and 4B). Teneligliptin also suppressed Ang II-induced expression of MCP-1 but not IL-6 in VSMC (Fig. 4C).

**Discussion**

In the present study, we showed that treatment with teneligliptin attenuated Ang II-induced AAA formation in ApoE KO mice. Hyperglycemia accelerates atherosclerosis and anti-diabetic agents are thought to exert anti-atherosclerotic effects by reducing serum glucose levels, which is associated with a reduction in oxidative stress levels\(^\text{21}\). Although atherosclerosis is involved in the development of AAAs, several studies showed that diabetes negatively correlated with AAA prevalence but the mechanisms are still unknown\(^\text{22}\). Tenelgliptin...
eral mechanisms for cholesterol-lowering effects of DPP-4 inhibitor have been suggested, which include suppression of intestinal secretion of triglyceride and cholesterol and de novo lipogenesis in the liver\textsuperscript{25}).

Recent studies have shown that macrophages infiltrating into the aortic wall play an important role in the development of AAAs\textsuperscript{12}). We showed that teneligliptin reduced macrophage infiltration into the aortic wall. It is difficult to determine the causal relationship between a reduction in macrophage infiltration and attenuation of aneurysm formation. However, our in vitro study showing that treatment with teneligliptin reduced Ang II-induced MCP-1 expression in VSMC.

High-fat diet-induced hypercholesterolemia is known to accelerate Ang II-induced AAA formation in ApoE KO mice\textsuperscript{23}). Because teneligliptin significantly reduces serum cholesterol levels, cholesterol-lowering effects may contribute to suppression of AAA formation. A meta-analysis revealed cholesterol-lowering effects of various DPP-4 inhibitors\textsuperscript{24)}, suggesting that it may be a class effect of DPP-4 inhibitors.

Fig. 3. Treatment with teneligliptin reduced macrophage infiltration into the aortic wall.

(A) A representative microphotograph of MAC2 staining of aortic section is shown in control mice (CTRL group, \(n = 11\)) and teneligliptin-treated mice (DPP-4i group, \(n = 12\)). The brown-colored cell indicates MAC2-positive cells. (B) The number of MAC2-positive cells were counted per section. *\(P < 0.05\) vs CTRL group.

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(A) Western blot analysis of phosphorylation of ERK and Akt in angiotensin (Ang) II (10^{-7} M)-stimulated VSMC in the presence or absence (CTRL) of teneligliptin (DPP-4i, 0, 10, 100 µM) treatment is shown. (B) The ratios of phosphorylated ERK/Akt to total ERK/Akt were quantified. N=5. *P<0.05. (C) mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-6 was determined by real-time qPCR in Ang II (10^{-7} M)-stimulated VSMC in the presence or absence (CTRL) of teneligliptin (DPP-4i, 100 µM) n=4. Expression of hypoxanthine phosphoribosyltransferase (Hprt) was used as an internal control to normalize the expression of IL-6 and MCP-1 *P<0.05 vs. CTRL without Ang II, #P<0.05 vs. CTRL+Ang II.

Fig. 4. Suppression of the response to angiotensin II by teneligliptin in vascular smooth muscle cells. (A) Western blot analysis of phosphorylation of ERK and Akt in angiotensin (Ang) II (10^{-7} M)-stimulated VSMC in the presence or absence (CTRL) of teneligliptin (DPP-4i, 0, 10, 100 µM) treatment is shown. (B) The ratios of phosphorylated ERK/Akt to total ERK/Akt were quantified. N=5. *P<0.05. (C) mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-6 was determined by real-time qPCR in Ang II (10^{-7} M)-stimulated VSMC in the presence or absence (CTRL) of teneligliptin (DPP-4i, 100 µM) n=4. Expression of hypoxanthine phosphoribosyltransferase (Hprt) was used as an internal control to normalize the expression of IL-6 and MCP-1 *P<0.05 vs. CTRL without Ang II, #P<0.05 vs. CTRL+Ang II.

suggests that reduction of macrophage recruitment by teneligliptin through MCP-1 suppression may contribute to attenuation of AAA formation. A previous study showed that treatment with DPP-4 inhibitors attenuated monocyte migration in response to MCP-1. Therefore, it may be possible that inhibition of MCP-1 production in VSMC and attenuation of monocyte chemotactic response to MCP-1 by treatment.
with DPP-4 inhibitors results in the reduction of macrophage recruitment and subsequent AAA formation.

Extracellular signal-regulated kinase plays an important role in the development of AAAs. A recent study showed that AAA formation is attenuated in ERK-1-deficient mice in an elastase-perfusion AAA model (27). It was reported that activation of ERK is involved in Ang II-induced MCP-1 expression in VSMCs (28). Therefore, it is reasonable to assume that inhibition of Ang II-induced ERK activation by teneligliptin is responsible for MCP-1 suppression. A previous study showed that alogliptin, another DPP-4 inhibitor, also suppressed lipopolysaccharide-induced ERK activation and MMP expression in U937 histiocytes (29), which may be consistent with our results. These studies suggest that suppression of ERK activation may play a central role in the attenuation of AAA formation by teneligliptin treatment through inhibition of MCP-1 and MMP expression. In addition to MCP-1, expression of plasminogen activator inhibitor-1 (30) and interleukin-6 (31) were reported to be activated by the Ang II/ERK pathway, suggesting that suppression of these molecules by teneligliptin may play a role in the suppression of AAA formation. However, the mechanism by which DPP-4 inhibitors suppress ERK activation is not clear and further studies are needed.

Akt is also reported to play an important role in the formation of AAA. A previous study showed that Akt was involved in the expression of pro-MMP9 and pro-MMP2 (32), suggesting the reduction of MMP availability through downregulation of Akt activation may play a role in the suppression of AAA formation by teneligliptin. However, there are conflicting results indicating that Akt activation plays a role in the protective effects of DPP-4 inhibitors (33, 34). Therefore, future studies are needed to confirm our in vitro findings can be replicated in vivo.

At this point, the mechanisms by which teneligliptin suppressed the Ang II-induced Akt/ERK pathway are not clear. A recent study showed that gemigliptin, another DPP-4 inhibitor, increased NF-E2 related factor 2 (Nrf2) expression in VSMCs (35). Nrf2 is a transcription factor that upregulates genes involved in antioxidative activities such as hemeoxygenase-1 and NAD(P)H quinone oxidoreductase. Therefore, it may be possible that teneligliptin suppressed Ang II-induced phosphorylation of Akt/ERK through reduction of reactive oxygen species. Although VSMC expressed DPP-4, the activation mechanism of Nrf2 by gemigliptin has not been determined. Further studies are needed to clarify the mechanisms of direct effects of DPP-4 inhibitors on VSMCs.

In addition to incretins, several other substrates for DPP-4 have been reported such as SDF-1α and natriuretic peptides (36). Considering that teneligliptin treatment did not affect serum glucose levels, it is plausible that an increase in the levels of these molecules may play a role in the attenuation of AAA development because SDF-1α induces recruitment of endothelial progenitor cells that are involved in the preservation of vascular integrity (36) and anti-inflammatory effects have been reported for natriuretic peptides (37).

In conclusion, we showed in the present study that inhibition of DPP-4 by teneligliptin attenuated Ang II-induced AAA formation possibly through MCP-1 suppression and macrophage recruitment. The present study and previous studies (16, 38) suggest that DPP-4 inhibitors may be novel therapeutics for the prevention of AAA formation. However, it remains to be determined whether DPP-4 inhibitors are effective in the prevention of further dilatation or rupture of already dilated aorta. Further studies are warranted.

Conflict of Interest

This study was partly supported by grants from Mitsubishi Tanabe Pharma Corporation (to Tokunou T and Ichiki T). Teneligliptin was provided by the Mitsubishi Tanabe Pharma Corporation.

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