Dipeptidyl-Peptidase-4 Inhibitor, Alogliptin, Attenuates Arterial Inflammation and Neointimal Formation After Injury in Low-Density Lipoprotein (LDL) Receptor-Deficient Mice

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Background—The results of recent studies suggest that dipeptidyl-peptidase-4 inhibitors have antiatherogenic effects. However, whether or not dipeptidyl-peptidase-4 inhibitors could suppress arterial inflammation and intimal hyperplasia after injury remains undetermined. The present study aims to clarify the anti-inflammatory effects of the dipeptidyl-peptidase-4 inhibitor, alogliptin (AGP), on the arteries of atherogenic low-density lipoprotein receptor-deficient (LKO) mice.

Methods and Results—We compared intimal hyperplasia in LKO mice 2 weeks after femoral artery injury using an external vascular cuff model. All mice received oral injection of AGP (20 mg/kg per day) or normal saline (control) once daily for 14 days. Fasting blood sugar levels, serum cholesterol levels, or blood pressure did not significantly differ between the 2 groups. Plasma levels of active glucagon-like peptide-1 were higher in the AGP than in the control LKO mice (22.2 ± 1.9 versus 15.6 ± 0.9 pg/mL; P < 0.05). Compared with saline, AGP significantly reduced intimal hyperplasia (1087 ± 127 versus 1896 ± 140 μm²; P < 0.001) as well as the intima/media ratio (0.08 ± 0.01 versus 0.16 ± 0.02; P < 0.001). Immunostaining showed that AGP reduced proliferating cell (proliferating cell nuclear antigen–positive nuclei; P < 0.001), percent smooth-muscle cell area (α-SMA-positive cells; P < 0.001), inflammatory cells infiltration (lymphocyte antigen 6 complex–positive cells; P < 0.05), tumor necrosis factor–α expression (P < 0.05), and percent phospho-NF-κB-positive cell compared with saline. Levels of tumor necrosis factor–α (0.5-fold P < 0.05), monocyte chemoattractant protein 1 (0.3-fold P < 0.01), and interleukin-1β (0.2-fold P < 0.05) mRNA were lower in the injured arteries of the AGP than in the control group.

Conclusions—AGP appeared to suppress neointimal formation by inhibiting inflammation, independently of its effects on glucose or cholesterol metabolism in atherogenic LKO mice. (J Am Heart Assoc. 2015;4:e001469 doi: 10.1161/JAHA.114.001469)

Key Words: cytokine • dipeptidyl-peptidase-4 inhibitor • inflammation • intimal hyperplasia • smooth muscle cell

Dipeptidyl peptidase-4 (DPP-4) inhibitors are novel oral antihyperglycemic agents for treating type 2 diabetes mellitus patients. Recent studies suggest that several DPP-4 inhibitors exert antiatherosclerotic effects though suppressing inflammatory reactions of monocytes and smooth-muscle cell (SMC) proliferation in vitro. However, whether or not DPP-4 inhibitors suppress arterial inflammation and intimal hyperplasia after injury remains undetermined.

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DPP-4 Inhibitor

The DPP-4 inhibitor, alogliptin benzonate (Takeda Pharmaceutical Company, Tokyo; 136 mg) was dissolved in distilled water to a final concentration of 1 mg/mL, stored at 4°C, and warmed to room temperature before use.

Femoral Artery Injury and AGP Administration

Only male mice were studied to exclude gender differences. The mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and we dissected the left femoral artery from its surrounding, as demonstrated previously. Vascular injury was inflicted by placing a nonocclusive polyethylene cuff (length 2 mm; internal diameter 0.56 mm; Becton Dickinson, Mountain View, CA) around the femoral artery. We administered AGP (20 mg/kg per day) or normal saline by oral injection once daily to the mice for 14 days.

Blood Pressure Measurement

Systolic blood pressure was measured in nonanesthetized mice at 13 days postinjury by the tail-cuff method (MK-2000; Muromachi Kikai).

Levels of Plasma Lipid, Glucose, and Active Glucagon-Like Peptide-1

Mice were fasted for 12 hours and blood samples were collected from the tail veins of both groups at 14 days postinjury. Levels of plasma total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were measured using high-performance liquid chromatography (Skylight Biotech Inc, Akita, Japan) as described previously. Levels of plasma fasting blood glucose were measured using Free Style Freedom (Nipro Co, Osaka, Japan). Levels of active glucagon-like peptide-1 (GLP-1) were measured in plasma from nonfasted mice using ELISA kits (Shibayagi Co, Gunma, Japan) at 14 days postinjury. Blood samples for measurement of active GLP-1 were immediately collected into tubes containing 1% AGP (10 mmol/L) vol/vol.

Arterial Harvest and Morphometric Analysis

After blood collection, the animals were euthanized by pentobarbital injection and the vascular tree was perfused with 0.9% NaCl followed by 4% paraformaldehyde. After the perfusion procedure, the femoral artery was harvested and fixed with 10% neutral-buffered formalin for 48 hours, embedded in paraffin, and sectioned (each 5-μm thickness). We used equally spaced (200-μm interval) 10 cross-sections to qualify a neointimal lesion for each mouse. The samples were stained with elastica van Gieson, and then photographed using an ECLIPS LV100 microscope (Nikon, Tokyo, Japan). The luminal, neointimal, and medial areas were calculated using NIH Image J 1.42 (National Institutes of Health, public domain software).

Immunohistochemistry

Smooth muscle cells were visualized using α-smooth muscle cell actin staining (N1584; DAKO, Tokyo, Japan), cell proliferation was investigated using proliferating cell nuclear antigen staining (N1529; DAKO, Tokyo, Japan), and inflammatory cells were detected by lymphocyte antigen 6 complex staining (N550291; BD Pharmingen in Japan, Tokyo, Japan). The proliferating cell nuclear antigen and the lymphocyte antigen 6 complex indexes were calculated as ratios of stained areas per total intimal area of injured arteries. In addition, anti-tumor necrosis factor-α (TNF-α) staining (ab-6671; Abcam, Cambridge, MA) was used to detect cytokine expressions and the extent of the TNF-α, and monocyte chemoattractant protein 1 (MCP-1) antibody (sc-1785; Santa Cruz Biotechnology) was used for detection of MCP-1 expression and evaluation for positive area per total intimal and medial area of injured arteries. Nuclear translocation of nuclear factor-κB (NF-κB) was detected by phospho-NF-κB p65 (ser276) (pNF-κB) staining (#3037; Cell Signaling Technology). Activation of pNF-κB was evaluated for percentage of positive nuclei in the intima and media or in the adventitia within a 50 μm radius from the external elastic lamina of injured arteries.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA from the injured femoral artery was isolated at 3 days postinjury using the TRI reagent (Sigma-Aldrich) (2 vessels for each sample). Complementary DNA was prepared from total RNA (500 ng) using reverse transcriptase according to the manufacturer’s instructions (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Quantitative mRNA expression was assessed by real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) using primers specific for TNF-α (forward: TCC CAG GTT CTC TTC AAG GGA, reverse: GGT GAG GAG CAC GTA TGC GG), MCP-1 (forward: CCT GGA TCG GAA CCA AAT GA, reverse: CGG GTC AAC TTC ACA TTC AAA G), interleukin (IL)-1β (forward: TGG GTG GTG ACG TTC CCA TT, reverse: CAG CAC GAG GCT TTT TTG TTG), and GAPDH (forward: GTC ATT GAG AGC AAC GCC AG, reverse: GTG TTC CTA CCG CCA ATG TG). Samples were run on the 7500 Fast Real-Time PCR system (Applied Biosystems). Data were analyzed by 7500 Software (Applied Biosystems), and relative expression levels of target mRNA of the AGP treatment group was compared with those of the control group.
Statistical Analysis

Statistical analyses were carried out using the Statistical Package for the Social Science (SPSS) software program, version 22.0 (SPSS Inc, Chicago, IL). Results are shown as the means±SE. Mean values of continuous variables were compared between groups using Student t test according to whether normally distributed as tested by the Shapiro–Wilk test, and P<0.05 was regarded as significant.

Results

LKO mice were treated with normal saline (control) or AGP for 14 days after cuff injury (Figure 1A). Blood pressure, fasting blood sugar, and serum cholesterol levels were similar at 2 weeks after cuff injury between the AGP- and saline-treated groups (Figure 1B). The nonfasting plasma levels of active GLP-1 were higher in mice treated with AGP than with saline (22.2±1.9 versus 15.6±0.9 pg/mL; P<0.05) (Figure 1C).

Neointimal Formation After Cuff Injury

We investigated the effect of AGP treatment on arterial inflammation in LKO mice after cuff-induced injury. Figure 2A shows representative cross-sections of femoral arteries harvested from AGP- and saline-treated LKO mice at 14 days postinjury. Quantitative analysis demonstrated that AGP significantly reduced the amount of intimal hyperplasia (1087±127 versus 1896±140 μm²; P<0.001) (Figure 2B) and the intima/media ratio (0.08±0.01 versus 0.16±0.02; P<0.001) (Figure 2C).

Proliferation of SMCs and Inflammatory Cells After Cuff Injury

We evaluated the effect of AGP on cell proliferation at 7 days after injury by staining for α-smooth muscle cell actin, proliferating cell nuclear antigen, and lymphocyte antigen 6 complex. The α-smooth muscle cell actin–positive areas in the neointima were significantly decreased in mice treated with

Figure 1. Study protocol, blood pressure, and blood sample findings at 14 days after injury. A, Schematic of study protocol. B, Systolic blood pressure (SBP) (AGP: n=9, control: n=8), fasting blood sugar levels (FBS) (AGP: n=7, control: n=8), and serum levels of non-HDL and HDL cholesterol (n=4 for each group) in mice at 14 days after injury. Data are expressed as means±SEM (n=4 to 9). C, Serum levels of active GLP-1 in AGP treated and control mice at 14 days after injury. Data are expressed as means±SEM (AGP: n=8, control: n=7), *P<0.05. AGP indicates alogliptin; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein; LDLR KO, low-density lipoprotein receptor knockout; NS, not significant.
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**Expression of Inflammatory Cytokine**

Figure 6 shows the expression of the proinflammatory cytokine TNF-α and MCP-1 after cuff injury determined using immuno-histochemistry and real-time PCR. Figure 6B shows the areas that expressed TNF-α and MCP-1 at 7 days after cuff injury in LKO mice. The percent positive area of TNF-α expression was significantly lower in the intima and media of mice treated with AGP than with saline (TNF-α: 1.4±0.6 versus 4.8±1.0%; P<0.05, MCP-1: 2.5±0.5 versus 5.4±0.4%; P<0.05) (Figure 6B).

Real-time PCR of the injured femoral artery revealed significantly decreased mRNA expression levels of TNF-α, MCP-1, and IL-1β in the AGP group compared with the control group (TNF-α: 0.49±0.05 versus 1.00±0.13; P<0.05, MCP-1: 0.34±0.06 versus 1.00±0.09; P<0.01, IL-1β: 0.16±0.03 versus 1.00±0.25; P<0.05) (Figure 6C).

**Evaluation of NF-κB Activation**

To examine postinjury NF-κB activation in the arteries of both AGP and control groups, we performed pNF-κB staining in the injured arteries of both groups at 7 days postinjury. Fewer pNF-κB-positive cells were observed in the intima and media (Figure 7A), and adventitia (Figure 7C) of AGP-treated mice compared with controls. The percentage of pNF-κB-positive cells in the arteries of the AGP group was significantly lower than in the control group (11.9±1.1 versus 20.6±1.7%; P<0.05) (Figure 7B). Furthermore, the percentage of pNF-κB-positive cells in the adventitia of the AGP group decreased by nearly half compared with the control group (18.0±4.4 versus 33.3±2.3%; P<0.05) (Figure 7D).

**Discussion**

This is the first investigation into how AGP affects injured arteries in vivo. AGP significantly reduced inflammation, SMC proliferation, and the TNF-α and MCP-1 levels in the injured arteries of LKO mice. Levels of fasting blood sugar and serum cholesterol did not significantly differ between AGP and control groups of LKO mice. These results suggest that AGP reduced neointimal formation by suppressing inflammation and smooth muscle cell activation.
Alogliptin (AGP) decreases numbers of inflammatory cells in the injured arteries of mice. A, Staining for lymphocyte antigen 6 complex (Ly-6G) in cuffed femoral arteries from mice at 7 days postinjury (bars=25 μm). Yellow arrows indicate the inflammatory cells and red arrows indicate internal elastic lamina, respectively. B, Quantitative analysis of Ly-6G-positive nuclei in the intima of cuffed arteries from mice at 7 days after injury. Data are expressed as means±SEM (n=3 for each group), *P<0.05.
A recent study has found that exendin-4 (GLP-1 receptor agonist) modulated monocyte adhesion to endothelial cells and attenuated atherosclerosis in mice and that these effects might contribute to the inhibition of p65 nuclear translocation in macrophages by means of cAMP levels that are increased by GLP-1 receptor activation.20 Another study found that exendin-4 suppresses SMC proliferation in arteries after wire injury.21 Furthermore, Matsubara et al showed that DPP-4 inhibitors and GLP-1 produce anti-inflammatory effects that are followed by increases in cytosolic cAMP levels and decreases in extracellular signal-regulated kinase (ERK) 1/2 and c-jun N-terminal kinase (JNK) phosphorylation as well as NF-κB activation in vitro.17

The cAMP/Protein kinase A (PKA) pathway attenuates TNF-α production in macrophages,22 and the present study found that AGP suppresses TNF-α expression in the injured artery. These findings suggest that GLP-1 levels increased by AGP significantly contribute to the anti-inflammatory effects of AGP though activating cAMP/PKA signaling. However, it is not clear whether the anti-inflammatory effects of DPP-4 inhibitors depend on GLP-1. Levels of active GLP-1 after AGP administration in the present study were much lower than those of the GLP-1 analog (exendin-4) that exerted anti-atherosclerogenic effect in another study21 (GLP-1 in our study: 5 to 7 pmol/L to GLP-1 analogue: 10 nmol/L), suggesting that DPP-4 may directly suppress atherosclerosis.

SMC proliferation participates in both the early and late phase of arterial diseases.23 NF-κB is a common regulator of proinflammatory genes. SMC proliferation and CARD11 mediate factor-specific activation of NF-κB.24 Interaction between DPP-4 and CARD11 leads to NF-κB activation in T-cells.25 Such activation was reduced by DPP-4 or CARD11 knockdown, and SMCs proliferation was dose-dependently attenuated by sitagliptin via suppressing NF-κB activation in vitro.16 In the present study, we show that AGP also attenuated SMC proliferation by suppressing NF-κB activation in vivo.

**Figure 6.** AGP decreases TNF-α expression in the injured arteries of LKO mice. A, Staining for TNF-α and MCP-1 in cuffed femoral arteries from AGP-treated (left) and control (right) mice at 7 days postinjury, respectively (bars=25 μm). Red arrows indicate internal elastic lamina. B, Quantitative analysis of TNF-α and MCP-1 positive areas in intima+media of AGP-treated and control mice at 7 days after injury. Data are expressed as means±SEM (n=3 for each group), *P<0.05. C, Bar graphs show the mRNA expression of TNF-α, MCP-1, and IL-1β in injured arteries from AGP-treated and control mice at 3 days after injury. Degree of change in gene expression is based on the mean amount of expression in control LKO mice. Data are expressed as means±SEM (n=4 per group), *P<0.05, **P<0.01. AGP indicates alogliptin; IL, interleukin; LKO, low-density lipoprotein receptor knockout; MCP-1, monocyte chemoattractant protein 1; TNF, tumor necrosis factor.

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The ERK pathway is also an important signaling cascade in inflammation. An in vitro study found that AGP reduced SMCs proliferation and attenuated ERK phosphorylation in SMCs, and that AGP reduces TNF-α production and NF-κB activation in monocytes. These findings suggest that inflammation and SMC proliferation might be suppressed by DPP-4 inhibitors directly, and not via GLP-1-dependent pathways.

DPP-4 inhibitors exert cardiovascular protective effects via other pathways. Several reports showed that AGP improved endothelial function through a GLP-1 independent pathway that includes NO release via the Src-Akt-eNOS pathway. Levels of stromal-derived factor-1α (SDF-1α), which is another physiological substrates of DPP-4, are increased by DPP-4 inhibitors and this might affect endothelial progenitor cells that mediate cardiovascular repair.

Levels of fasting blood sugar levels and serum cholesterol did not significantly differ between AGP- and saline-treated LKO mice. These findings are consistent with those of a study showing that AGP administration for 12 weeks did not change blood pressure or the lipid and glycemic profiles in LKO mice with a diet containing normal amounts of fat. Clinical data also suggest that AGP exerts minor effects on lipid profiles.

In conclusion, AGP suppressed intimal hyperplasia caused by vascular inflammation such as atherosclerosis and helped to reduce the frequency of cardiovascular events.

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Disclosures

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