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DPP-4 (CD26) Inhibitor Alogliptin Inhibits Atherosclerosis in Diabetic Apolipoprotein E-Deficient Mice

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Abstract

Dipeptidyl peptidase-4 (DPP-4 or CD26) inhibitors, a new class of anti-diabetic compounds, are effective in treatment of hyperglycemia. Since atherosclerosis-related cardiovascular diseases are the major complications of diabetes, it is important to determine the effect of DPP-4 inhibitors on atherosclerosis. In this study, nondiabetic and diabetic apolipoprotein E (apoE)-deficient mice were treated with DPP-4 inhibitor alogliptin for 24 weeks and atherosclerotic lesions in aortic origins were examined. Results showed that diabetes significantly increased atherosclerotic lesions, but alogliptin treatment reduced atherosclerotic lesions in diabetic mice. Metabolic studies showed that diabetes increased plasma glucose and alogliptin treatment reduced glucose. Furthermore, immunohistochemistry study showed that diabetes increased IL-6 and IL-1 β protein expression in atherosclerotic plaques, but alogliptin treatment attenuated diabetes-augmented IL-6 and IL-1 β expression. In consistence with the observations from the mouse models, our in vitro studies showed that alogliptin inhibited toll-like receptor (TLR)4-mediated upregulation of IL-6, IL-1 β , and other proinflammatory cytokines by mononuclear cells. Taken together, our findings showed that alogliptin inhibited atherosclerosis in diabetic apoE-deficient mice and the actions of alogliptin on both glucose and inflammation may contribute to the inhibition.

Keywords

Dipeptidyl peptidase-4; CD26; Atherosclerosis; Diabetes; Inflammation

INTRODUCTION

Dipeptidyl peptidase-4 (DPP-4 or CD26) cleaves multiple peptide substrates, including the incretin hormones glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP) that stimulate insulin secretion from β -cells and inhibit hepatic glucose production (1). Clinical studies have shown that inhibitors of DPP-4 activity lower fasting and postprandial glucose levels and improve β -cell function, indicating that DPP-4 is a validated target for the treatment of type 2 diabetes (2). In addition to the anti-diabetes

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property, recent studies have suggested that DPP-4 inhibitors also have anti-inflammatory effects. Ferreira et al. reported that sitagliptin, a DPP-4 inhibitor, reduced C-reactive protein and interleukin 1 (IL-1) β in diabetic animal models (3). Our group found that alogliptin inhibited the expression of matrix metalloproteinases (MMPs), a group of powerful proteinases involved in the tissue degradation and tissue remodeling in inflammatory diseases (4). Furthermore, Jungraithmayr et al. reported that DPP-4 inhibitor attenuated ischemia/reperfusion injury in mouse lung transplants (5). Given that DPP-4 is identical to CD26 (6), a cell surface glycoprotein with multiple functions in T cell activation, DNA synthesis, cell proliferation, cytokine production and signaling activation (7-9), it is likely that inhibition of DPP-4 (CD26) modulates inflammatory processes.

Atherosclerosis-associated cardiovascular diseases are the major complications of diabetes and inflammation plays a major role in atherosclerosis. However, the impact of DPP-4 inhibition on atherosclerosis in animal models has not been well elucidated. In this study, we have investigated the effect of alogliptin on atherosclerosis in nondiabetic and diabetic apolipoprotein E-deficient (apoE^{-/-}) mice.

MATERIALS AND METHODS

Animals, diets and drug treatments

Four weeks old male apoE^{-/-} mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at the animal facility of Medical University of South Carolina. Since female apoE-deficient mice are resistant to STZ treatment (10), only male mice were used in this study. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC). All mice were maintained on a 12-hour light-dark cycle in a pathogen-free environment and had ad libitum access to water. One week after arrival, high-fat diet (D12492, Research Diets, New Brunswick, NJ), in which 60% of kilocalories are from fat, was provided to all mice. Mice were randomly divided into 4 groups (n=10 per group). At age 12 weeks, mice in Groups 3 and 4 were rendered diabetic by intraperitoneal administration of single dose of STZ (200 mg/kg in citrate buffer, 0.05 mol/L; pH 4.5 (Sigma Aldrich, St. Louis, MO). Four weeks after STZ treatment, mice in Groups 2 and 4 were treated with alogliptin (15 mg/kg) provided by Takeda Pharmaceuticals Inc. (Deerfield, IL) by daily gavaging through a syringe with a curved and end-dulled needle for 24 weeks.

Metabolic measurements

Blood samples were obtained under the fasted condition and glucose level was determined using a Precision QID glucometer (MediSense Inc., Bedford, MA). Plasma insulin level was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Metuchen, NJ). Plasma cholesterol and triglycerides were measured using enzymatic methods (Wako Diagnostics, Richmond, VA).

Histological analysis of atherosclerotic lesions

The tissues of aortic root were frozen in Tris-buffered saline (TBS) freezing media and sectioned with a cryostat. Sections with 5 μ thickness were cut and mounted on slides before being placed in 95% ethanol for 10 minutes and being washed in phosphate-buffered saline (PBS). Fixed hydrated slides were stained with Harris modified hematoxylin (Fisher Scientific, Pittsburgh, PA) for 3 minutes and then rinsed in deionized water. Staining of hematoxylin was then developed in tap water for 5 minutes and dipped in acid ethanol to stop the development of the staining. Slides were stained with eosin in Harleco 1% alcohol solution (EMD Chemicals, Gibbstown, NJ) for 30 seconds. Slides were placed in Coplin jars with 95% ethanol three times for 5 minutes each and then repeated in 100% ethanol. Slides were further dehydrated in 99.5% xylene (Sigma Aldrich). Slides were mounted in xylene

based Cytoseal-XYL mounting media (Fisher Scientific). Photomicrographs of tissue sections were taken using a Nikon Eclipse 90i digital microscope with the NIS-Elements 3.10. Image Analysis System (Nikon Instruments Inc., Lewisville, TX). The area of intima was expressed as the percentage of the total aortic area including intima, media, and lumen (11).

Immunohistochemical analysis of gene expression

Sections were blocked with 2% normal goat serum for 20 minutes. Primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were applied at 1:40-1:100 dilutions for 45 minutes. Sections were then incubated with secondary biotinylated-antibody from the ABC Elite kit (Vector Laboratories, Burlingame, CA) for 30 minutes at 1:250 dilutions. Sections were blocked with avidin-biotin solution from the ABC Elite kit for 30 minutes. Slides were then placed in 30% methanol peroxide. After each step, slides were rinsed twice in PBS, pH 7.4 for 3 minutes. Slides were covered with diaminobenzidine peroxidase substrate solution from the Impact DAB kit (Vector Laboratories) for 3 minutes and then rinsed in water. Counterstaining was performed with hematoxylin. Slides were then dehydrated using increasing concentrations of ethanol and xylenes and mounted. Staining with normal rabbit IgG was used as a negative control. Positively immunostained areas were observed and analyzed using the computer-based image analysis system as described above for the histological analysis.

Image analysis

Images were analyzed with the Photoshop software (version 10; Adobe Systems, San Jose, CA). The method to use “Similar” feature to select a particular color staining on a digitized immunohistochemical image has previously been described in detail (12). Briefly, a standard was created by selecting an area of 0.5 cm × 0.5 cm from a tissue section that had desired brown color from immunostaining. The cursor of the Magic Wand tool was clicked on the standard to make a selection and the area of the standard was highlighted. To specify how broad a range of color the Magic Wand tool should include in the selection, the Tolerance value in the Magic Wand Options palette was set to 100. Using the “Similar” command, all the areas with the brown color that is similar to the standard on an image being determined were highlighted. The quantification was done using the Histogram command in the Image menu, which showed the pixels of the highlighted area. The degree of the immunostaining was presented as average pixels per atherosclerotic plaque.

Cell culture

Human U937 mononuclear cells (13) were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in a 5% CO₂ atmosphere in RPMI 1640 medium (GIBCO, Invitrogen Corp., Carlsbad, CA) containing 10% fetal calf serum, 1% MEM non-essential amino acid solution, 0.6 g/100 ml of HEPES and 5 mM of glucose. The medium was changed every 2-3 days. Human aortic endothelial cells and culture medium were purchased from Invitrogen Corp. and cultured by following the instruction from the manufacturer.

Cell treatment—U937 cells were treated with or without 10 ng/ml of LPS (Sigma Aldrich) in the absence or presence of 0.5, 1, or 5 nM of alogliptin for 24 h. After the treatment, IL-6 and IL-1 β in culture medium was quantified using ELISA and cellular IL-6 mRNA was quantified using real-time PCR. Human aortic endothelial cells were treated with or without 10 ng/ml of LPS (Sigma Aldrich) in the absence or presence of 0.5, 1, or 5 nM of alogliptin for 24 h. After the treatment, IL-6 in culture medium was quantified using ELISA.

ELISA

IL-6 and IL-1 β in conditioned medium was quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R&D System, Minneapolis, MN).

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using the RNeasy minikit (Qiagen, Santa Clarita, CA). First-strand complementary DNA (cDNA) was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed with primers (5' primer sequence, AACAACTGAACCTTCCAAAGATG; 3' primer sequence, TCAAACTCCAAAAGACCAGTGATG) to quantify IL-6 mRNA as described (14, 15). As control, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was also quantified with the primers (5' primer sequence, GAATTTGGCTACAGCAACAGG GTG; 3' primer sequence, TCTCTTCCTCTTGCTCTTGCTG).

PCR array

The first strand cDNA was synthesized from RNA using the RT² First Strand Kit (SABioscience Corp., Frederick, MD). Human common cytokine PCR array (Catalog # PAHS-021, SABioscience Corp.) was used to profile cytokine expression according to the instructions from the manufacturer.

Statistic analysis

Data were presented as mean $\pm$ SD. Student *t* tests were performed to determine the statistical significance of differences of intimal lesion size and gene expression among different experimental groups. A value of *P* < 0.05 was considered significant.

RESULTS

Effects of alogliptin on metabolic parameters

At the end of the study, the effects of alogliptin treatment on metabolic parameters including body weight, insulin, glucose, cholesterol and triglycerides were determined. Results showed that the induction of diabetes in apoE^{-/-} mice decreased body weight, which is consistent with the previous reports (16, 17), but alogliptin treatment had no effect on body weight (Fig. 1A). Treatment of mice with STZ reduced insulin level (Fig. 1B), increased glucose level as expected, and decreased triglycerides (Table 1). Alogliptin significantly lowered STZ-increased glucose level (Table 1). Interestingly, alogliptin treatment also decreased cholesterol and triglycerides in diabetic apoE^{-/-} mice as compared with those in nondiabetic mice (Table 1).

The glucose and lipids were also measured at 12 weeks of alogliptin treatment. The results showed that the glucose and cholesterol levels measured at 12 weeks were much lower than those measured at 24 weeks in mice without alogliptin treatment (Table 1), indicating a marked increase in glucose and lipid levels in the next 12 weeks by high-fat diet feeding. Furthermore, data showed that diabetes increased glucose and triglycerides, and decreased cholesterol at 12 weeks. Alogliptin treatment reduced glucose and triglycerides, but not cholesterol, at 12 weeks of alogliptin treatment.

Alogliptin inhibits atherosclerosis in diabetic apoE^{-/-} mice

At the end of the study, the vascular tissues in the aortic origin of all mice were frozen sectioned and analyzed with H/E staining. Results showed that diabetic mice had significantly larger intimal lesions than nondiabetic mice (*p* < 0.01) (Figs. 2A and 2B).

Results also showed that alogliptin treatment had no effect on lesion size in nondiabetic mice, but significantly reduced lesion size in diabetic mice (Figs. 2A and 2B).

Alogliptin inhibits IL-6 and IL-1 β expression in atherosclerotic plaques in diabetic apoE $^{-/-}$ mice

To determine the effect of alogliptin on vascular inflammation, we focused on IL-6 (Figs. 3A and 3B) and IL-1 β (Figs. 4A and 4B) because these cytokines play an important role in atherosclerosis (18). Results showed that IL-6 and IL-1 β expression in atherosclerotic plaques was higher in diabetic mice than that in nondiabetic mice and alogliptin treatment led to a significantly decreased IL-6 and IL-1 β expression in diabetic, but not nondiabetic mice.

Alogliptin inhibits IL-6 expression by mononuclear cells in vitro

The effect of alogliptin on IL-6 expression by mononuclear cells was further studied with cultured U937 mononuclear cells. Results showed that alogliptin inhibited IL-6 secretion from U937 mononuclear cells stimulated with LPS, a ligand for toll-like receptor (TLR)4 and a potent stimulator for proinflammatory cytokine expression (19), in a concentration-dependent manner with a maximal 55% inhibition at 1 nM (Fig. 5A). Alogliptin also inhibited LPS-stimulated IL-6 mRNA expression (Fig. 5B), indicating that the inhibition of IL-6 secretion by alogliptin is due to the inhibition of IL-6 expression. In addition to IL-6, alogliptin also inhibited the secretion of IL-1 β (Fig. 5C), another proinflammatory cytokine. Furthermore, gene expression analysis from the PCR array studies showed that besides IL-6 and IL-1 β , alogliptin inhibited the expression of a number of proinflammatory cytokines such as interferon alpha (IFNA1) and lymphotoxin alpha and beta (LTA and LTB) (Table 2).

The effect of alogliptin on IL-6 expression by aortic endothelial cells has been also studied and the results showed that alogliptin had no effect on LPS-stimulated IL-6 secretion (Fig. 6), suggesting that the effect of alogliptin on IL-6 expression by mononuclear cells is cell type-specific.

DISCUSSION

Clinical studies have shown that glucose- or lipid-lowering drugs prescribed to patients with type 2 diabetes such as pioglitazone, statins and fibrates reduce cardiovascular events in diabetic patients (20-22). Since these drugs not only reduce plasma glucose or lipids, but also inhibit inflammation, it is believed that they protect cardiovascular system via both glucose-dependent and -independent mechanisms. In recent years, DPP-4 inhibitors, a new class of anti-diabetes compounds, have been developed to treat patients with type 2 diabetes. DPP-4 inhibitors reduce glucose level by inhibiting DPP-4-mediated inactivation of incretin hormones such as GLP-1 and GIP that stimulate insulin secretion from β -cells and inhibit hepatic glucose production (1). Although DPP-4 inhibitors have been shown to be effective in treatment of hyperglycemia, their effects on atherosclerosis and vascular inflammation have not been well elucidated.

Alogliptin is a potent, selective, and bioavailable inhibitor of DPP-4 (23). Clinical trials have shown that alogliptin is effective in treatment of hyperglycemia in patients with type 2 diabetes (24, 25). In consistence with the patient study, our study showed that alogliptin reduced fasting glucose in diabetic apoE $^{-/-}$ mice. A recent report by Zhang et al. showed that alogliptin treatment reduced both postprandial and fasting blood glucose levels in a dose-dependent manner in mice with diabetes induced by high-fat diet feeding and STZ injection (26). This study also reported that the plasma level of alogliptin was 26.1 ± 7.2 ng/ml in

mice treated with alogliptin at the dose of 15 mg/kg. Since it has been shown that the plasma concentration of alogliptin required to produce 50% inhibition (EC₅₀) of DPP-4 activity in diabetic patients is 3.7 ng/ml (27), the above plasma level of alogliptin is sufficient to achieve a significant inhibition of DPP-4 activity. To elucidate the mechanism by which alogliptin reduced plasma glucose in animal model, Moritoh et al. have shown that alogliptin increased plasma active GLP-1 by 4 folds in ob/ob mice (28).

In this study, we employed a mouse model with STZ-induced diabetes. Since one key mechanism by which DPP-4 inhibitors lower glucose level is to increase insulin production from β cells by increasing incretin, it is important that partial β cells in pancreas are preserved. Thus, we only treated mice with one single STZ injection. Indeed, insulin test showed that diabetic mice had 22% of insulin level in nondiabetic mice. Although the study by Zhang et al. showed that alogliptin restored the β -cell mass and islet morphology, and increased insulin secretion in their diabetic mouse model induced by STZ treatment, which was similar to the model used in our current study (26), it is possible that the preserved β cells might have been injured and thus have decreased response to incretin, which may lead to less effectiveness in glucose lowering by alogliptin.

We found in this study that alogliptin treatment reduced atherosclerotic lesions and inhibited IL-6 and IL-1 β expression in atherosclerotic lesions in diabetic mice. To understand the potential mechanisms by which alogliptin reduced atherosclerosis in diabetic mice, we first noticed that the diabetic mice had a significant increase in glucose, but not cholesterol, when compared with nondiabetic mice, and alogliptin decreased glucose in diabetic mice. Thus, it is likely that alogliptin reduced atherosclerosis via a glucose-dependent mechanism.

The role of hyperglycemia in atherosclerosis in diabetic mice has been reported previously by a large number of studies (29). Most of the studies showed that STZ-induced hyperglycemia was accompanied by an increase in total cholesterol and triglycerides (30), which made it difficult to interpret the role of hyperglycemia in atherosclerosis. However, several studies reported that STZ treatment significantly increased glucose, but not cholesterol and triglycerides. For examples, Koh et al. showed that induction of diabetes in apoE^{-/-} mice was associated with increased glucose and glycated hemoglobin levels, and a 5-fold increase in aortic atherosclerosis (31). They also showed that serum levels of total cholesterol, LDL, HDL and triglycerides were not significantly altered by diabetes. Hayek et al. and Tse et al. also reported previously that STZ treatment resulted in a significant increase in glucose, but not cholesterol and triglycerides, and accelerated atherosclerosis in apoE^{-/-} mice (32, 33). Although it remains unclear why no significant increase in cholesterol and triglycerides were observed along with increased glucose by STZ-treatment in these studies, their findings suggest a role of hyperglycemia in atherosclerosis.

In our study, all mice were fed high-fat diet and hence nondiabetic mice had high levels of cholesterol and triglycerides (Table 1). It is possible that the high levels of cholesterol and triglycerides in mice prevented further increase in cholesterol and triglycerides by induction of diabetes. This is likely the reason why no increases in cholesterol and triglycerides were observed in diabetic mice in our study. Thus, our study indicated that hyperglycemia played an important role in atherosclerosis in diabetic apoE^{-/-} mice. In addition to the above observations, we also found that alogliptin treatment in diabetic mice was associated with a decrease in not only glucose, but also cholesterol and triglycerides (Table 1), suggesting a relationship between glucose and lipid metabolism in apoE^{-/-} mice. By reducing both glucose and lipids, it is not surprising to find that the atherosclerotic lesion size in alogliptin-treated diabetic mice was even smaller than that in nondiabetic mice (Fig. 2).

Our study showed that alogliptin lowered glucose in diabetic, but not nondiabetic mice. This observation might be explained by glucose-dependent action of incretin such as GLP-1 on the stimulation of insulin secretion from β cells. Werner et al. have shown that GLP-1 receptor agonist improves glucose-stimulated insulin secretion in a strictly glucose-dependent manner (34). Since glucose was not increased in the nondiabetic mice, increase in GLP by alogliptin treatment would not significantly increase insulin secretion and thus decrease glucose.

Our data showed that alogliptin not only reduced glucose, but also inhibited IL-6 and IL-1 β expression in atherosclerotic lesions (Figs. 3 and 4), suggesting that in addition to the glucose-dependent mechanism, alogliptin may also inhibit atherosclerosis by targeting vascular inflammation through a glucose-independent mechanism. We focused on IL-6 and IL-1 β expression in this study since these proinflammatory cytokines have been shown by many studies to be associated with atherosclerosis. A recent clinical study in 306 patients with type 2 diabetes showed that serum IL-6, but not C-reactive protein, was associated with coronary artery calcium score that has been used to predict the future events associated with coronary heart disease (18). Another clinical study in 226 patients without cerebrovascular disease showed that increased levels of IL-6 were associated with subclinical intracranial large-artery atherosclerosis detected by magnetic resonance angiography (35). Furthermore, in apoE $^{-/-}$ mice, it has been shown that the secretion of IL-6 from isolated aortas was significantly correlated with atherosclerotic lesion area (36). Our data from this study showed that diabetes increased IL-6 expression in atherosclerotic lesions significantly, but alogliptin inhibited diabetes-increased IL-6 expression (Fig. 3). Given the crucial role of IL-6 in atherosclerosis, it is likely that the inhibition of IL-6 expression by alogliptin contributed to alogliptin-inhibited atherosclerosis in diabetic mice.

In support of the above observation that IL-6 and IL-1 β expression was inhibited in atherosclerotic lesions by alogliptin, our in vitro studies showed that alogliptin inhibited IL-6 and IL-1 β expression induced by LPS, a ligand for TLR4 (Fig. 4), by U937 mononuclear cells. Recent studies by Jialal and coworkers showed that patients with diabetes had higher TLR4 and TLR2 surface expression on monocytes and more TLR4 and TLR2 ligands in serum than nondiabetic patients (37). Furthermore, the role of TLR4 and TLR2 in atherosclerosis has been well documented by animal studies (38). Thus, TLR4 and TLR2 are likely to play an essential role in increased atherosclerosis in diabetic mice and it is interesting to find out that alogliptin inhibits TLR4-mediated IL-6 and IL-1 β expression.

Our in vitro studies showed that alogliptin only inhibited LPS-stimulated proinflammatory cytokine expression, but not the baseline of cytokine expression. Since LPS is a potent ligand for TLR4, these findings suggest that alogliptin specifically targets TLR4-mediated inflammatory response. Based on these observations, we hypothesized that in nondiabetic apoE-deficient mice, the TLR4-mediated inflammatory response was not as strong as that in diabetic apoE-deficient mice, and alogliptin hence does not have a significant effect. In contrast, diabetes markedly enhanced TLR4-mediated inflammatory response in diabetic mice and alogliptin treatment thus has significant effect on inflammatory parameters and atherosclerosis. Indeed, our previous studies have shown that high glucose remarkably increased TLR4-mediated inflammatory response in mononuclear cells (39, 40).

Several recent reports support the anti-inflammatory functions of DPP-4 inhibitors. A study showed that treatment of Zucker Diabetic Fatty (ZDF) rats, an animal model of obese type 2 diabetes, with sitagliptin reduced glucose, HbA1C, total cholesterol, triglycerides, CRP and proinflammatory cytokine IL-1 β (3). Our group reported that alogliptin inhibited toll-like receptor (TLR4)-mediated extracellular signal-regulated kinase (ERK) signaling activation and ERK-dependent MMP expression by mononuclear cells (4). Furthermore, it has been

reported that treatment of orthotopic mouse lung transplants with DPP-4 inhibitor resulted in significant improvement of gas exchange, less lipid oxidation, preservation of parenchymal ultrastructure, reduced neutrophil infiltration, and reduced myeloperoxidase expression, leading to a less ischemia/reperfusion injury and better lung function and structure integrity (5).

To understand the potential actions of DPP-4 inhibition, it is noteworthy that CD26, a 110-kDa cell surface glycoprotein, has intrinsic DPP-4 activity (6). CD26 is expressed by a variety of cells including T cells, B cells, NK cells, and macrophages (7, 8, 41) and has multiple functions in T cell activation, DNA synthesis, cell proliferation, cytokine production and signaling activation (7-9). Given the role of CD26 in inflammation, DPP-4 inhibition may modulate CD26 activities in T cells and macrophages and affect the development of atherosclerosis.

In conclusion, our animal study demonstrated for the first time that DPP-4 inhibitor alogliptin inhibited atherosclerosis and expression of IL-6 and IL-1 β in atherosclerotic lesions in diabetic apoE^{-/-} mice. Our in vitro study also demonstrated that alogliptin inhibited expression of proinflammatory cytokines by mononuclear cells.

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Figure 1.

The effects of alogliptin on body weight (A) and the effect of STZ treatment on insulin level (B). The body weight was determined after nondiabetic and diabetic apoE^{-/-} mice were treated with or without 15 mg/kg/day of alogliptin for 24 weeks. The plasma insulin levels were determined in mice treated without or with STZ. The data presented are mean $\pm$ SD of 7-9 mice in each group.

Figure 2.

A, Representative photomicrographs of atherosclerotic lesions in the aortic origins of nondiabetic or diabetic apoE^{-/-} mice treated with or without 15 mg/kg/day of alogliptin for 24 weeks. Diabetes was induced by STZ treatment and all mice were fed high-fat diet. B, Quantification of intimal lesion area of atherosclerotic plaques in aortic origins of apoE^{-/-} mice (n=7-9). The area of the intimal lesions was presented as percentage of the total aortic area including intima, media, and lumen.

Figure 3.

A, IL-6 protein expression in aortic atherosclerotic plaques of nondiabetic or diabetic apoE^{-/-} mice treated with or without 15 mg/kg/day of alogliptin. Mice were sacrificed after the treatment and aortic origins were dissected and processed for immunohistochemical analysis as described in Methods. B, Quantification of IL-6 protein expression in atherosclerotic plaques (n=7-9). IL-6 detected by immunostaining was quantified as described in the Methods.

B.

Figure 4.

A, IL-1 β protein expression in aortic atherosclerotic plaques of nondiabetic or diabetic apoE^{-/-} mice treated with or without 15 mg/kg/day of alogliptin. Mice were sacrificed after the treatment and aortic origins were dissected and processed for immunohistochemical analysis as described in Methods. B, Quantification of IL-1 β protein expression in atherosclerotic plaques (n=6-9). IL-1 β detected by immunostaining was quantified as described in the Methods.

Figure 5.

Inhibition of LPS-stimulated proinflammatory cytokine secretion and mRNA expression in U937 cells by alogliptin. A and C, U937 cells were treated with or without 10 ng/ml of LPS in the absence or presence of different concentrations of alogliptin for 24 h. After the treatment, IL-6 (A) and IL-1 β (C) in culture medium were quantified using ELISA. B, U937 cells were treated with or without 10 ng/ml of LPS in the absence or presence of 1 nM of alogliptin for 24 h. After the treatment, cellular IL-6 mRNA was quantified using real-time PCR. The expression of IL-6 mRNA was normalized to that of GAPDH mRNA.

Figure 6.

The effect of alogliptin on LPS-stimulated IL-6 secretion by human aortic endothelial cells. Human aortic endothelial cells were treated with or without 10 ng/ml of LPS in the absence or presence of different concentrations of alogliptin for 24 h. After the treatment, IL-6 in culture medium was quantified using ELISA.

Table 1
Metabolic Parameters for Nondiabetic and Diabetic Mice Treated with or without alogliptin

Diabetes status	Alogliptin treatment	Glucose (mg/dl)		Cholesterol (mg/dl)		Triglycerides (mg/dl)	
		12 weeks	24 weeks	12 weeks	24 weeks	12 weeks	24 weeks
Non-diabetic	No	165 ± 16 ^a	255 ± 5 ^d	293 ± 23	918 ± 94	80 ± 5 ⁱ	185 ± 12 ^l
	Yes	161 ± 23	308 ± 31	344 ± 114	723 ± 115	131 ± 4	162 ± 20
Diabetic	No	255 ± 83 ^b	473 ± 51 ^e	213 ± 82	736 ± 86 ^g	190 ± 34 ^j	148 ± 20 ^m
	Yes	129 ± 71 ^c	349 ± 55 ^f	213 ± 31	537 ± 77 ^h	71 ± 5 ^k	106 ± 16 ⁿ

The baseline levels of glucose, cholesterol and triglyceride at 0 week were 174 ± 14, 178 ± 23, 117 ± 38 mg/dl, respectively. Fasting glucose, cholesterol and triglycerides were measured at 12 or 24 weeks after treatment of nondiabetic and diabetic mice with alogliptin at the dose of 15 mg/kg/day.

Glucose: ^a $p < 0.05$ vs ^c, ^c $p < 0.01$ vs ^b, ^e $p < 0.01$ vs ^d, ^f $p < 0.05$ vs ^e; Cholesterol: ^h $p < 0.05$ vs ^g; Triglycerides: ^j $p < 0.01$ vs ⁱ, ^k $p < 0.01$ vs ^j, ^m $p < 0.05$ vs ^j, ⁿ $p < 0.05$ vs ^m.

Table 2

The effect of alogliptin on LPS-stimulated expression of cytokines

Genes	Control cells	Cells treated with LPS	Cells treated with LPS and alogliptin	% inhibition by alogliptin on LPS-stimulated gene expression
IL-1 α	1.00 (27.5)	2.00 (26.5)	1.32 (27.1)	68%
IL-1 β	1.00 (27.3)	2.00 (26.3)	1.23 (27.0)	77%
IL-1F10 (interleukin 1 family, member 10)	1.00 (29.2)	2.64 (27.8)	1.41 (28.7)	75%
IL-1F6 (interleukin 1 family, member 6)	1.00 (28.0)	2.30 (26.8)	1.23 (27.7)	82%
IL-2	1.00 (29.8)	2.64 (28.4)	1.41 (29.3)	75%
IL-3	1.00 (30.1)	2.00 (29.1)	0.76 (30.6)	100%
IL-5	1.00 (29.6)	2.30 (28.4)	1.32 (29.2)	75%
IL-6	1.00 (28.7)	2.00 (27.7)	1.32 (28.3)	68%
IL-13	1.00 (29.7)	1.62 (29.0)	0.76 (30.1)	100%
IL-17A	1.00 (29.1)	2.46 (27.8)	1.07 (29.0)	95%
IL-17C	1.00 (28.6)	1.87 (27.7)	1.00 (28.6)	100%
IL-19	1.00 (27.8)	2.30 (26.6)	1.00 (27.8)	100%
IL-20	1.00 (27.5)	2.00 (26.5)	1.00 (27.5)	100%
IL-21	1.00 (28.1)	2.30 (26.9)	1.23 (27.8)	82%
IL-22	1.00 (30.2)	3.24 (28.5)	1.23 (29.8)	90%
IFNA1 (interferon alpha 1)	1.00 (29.1)	2.00 (28.1)	1.07 (29.0)	93%
IFNA5 (interferon alpha 5)	1.00 (28.4)	2.46 (27.1)	1.07 (28.3)	95%
LTA (lymphotoxin alpha)	1.00 (28.3)	2.46 (27.0)	1.23 (28.0)	84%
LTB (lymphotoxin beta)	1.00 (30.9)	2.14 (29.8)	1.07 (30.8)	94%
PDGFA (platelet-derived growth factor alpha polypeptide)	1.00 (30.1)	2.00 (29.1)	1.32 (30.5)	68%
TNFRSF11B (tumor necrosis factor receptor superfamily, member 11B)	1.00 (29.6)	2.46 (28.3)	1.32 (29.2)	78%
TNFSF12 (tumor necrosis factor superfamily, member 12)	1.00 (29.2)	2.30 (28.0)	1.23 (28.9)	82%

The expression of cytokines in control cells was designated as 1.00. The expression of cytokines by treatment with LPS or LPS plus alogliptin was presented as fold of the control. The cycle thresholds (*C_t*) are presented in the parentheses. The % inhibition by alogliptin on LPS-stimulated cytokine expression was calculated by the following equation: (Increased fold in cells treated with LPS alone - increased fold in cells treated with LPS and alogliptin)/Increased fold in cells treated with LPS alone $\times$ 100.