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Long-Term Dipeptidyl-Peptidase 4 Inhibition Reduces Atherosclerosis and Inflammation via Effects on Monocyte Recruitment and Chemotaxis

Zubair Shah, MD; Thomas Kampfrath, PhD; Jeffrey A. Deiuliis, PhD; Jixin Zhong, MD, PhD; Colleen Pineda, MS; Zhekang Ying, MD, PhD; Xiaohua Xu, PhD; Bo Lu, PhD; Susan Moffatt-Bruce, MD, PhD; Rekha Durairaj, MD; Qinghua Sun, MD, PhD; Georgeta Mihai, PhD; Andrei Maiseyeu, PhD; Sanjay Rajagopalan, MD

Background—Dipeptidyl-peptidase 4 (DPP-4) inhibitors are increasingly used to accomplish glycemic targets in patients with type II diabetes mellitus. Because DPP-4 is expressed in inflammatory cells, we hypothesized that its inhibition will exert favorable effects in atherosclerosis.

Methods and Results—Male LDLR\(^{-/-}\) mice (6 weeks) were fed a high-fat diet or normal chow diet for 4 weeks and then randomized to vehicle or alogliptin, a high-affinity DPP-4 inhibitor (40 mg·kg\(^{-1}\)·d\(^{-1}\)), for 12 weeks. Metabolic parameters, blood pressure, vascular function, atherosclerosis burden, and indexes of inflammation were obtained in target tissues, including the vasculature, adipose, and bone marrow, with assessment of global and cell-specific inflammatory pathways. In vitro and in vivo assays of DPP-4 inhibition (DPP-4i) on monocyte activation/migration were conducted in both human and murine cells and in a short-term ApoE\(^{-/-}\) mouse model. DPP-4i improved markers of insulin resistance and reduced blood pressure. DPP-4i reduced visceral adipose tissue macrophage content (adipose tissue macrophages; CD11b\(^{+}\), CD11c\(^{+}\), Ly6C\(^{hi}\)) concomitant with upregulation of CD163. DPP-4 was highly expressed in bone marrow–derived CD11b\(^{+}\) cells, with DPP-4i downregulating proinflammatory genes in these cells. DPP-4i decreased aortic plaque with a striking reduction in plaque macrophages. DPP-4i prevented monocyte migration and actin polymerization in in vitro assays via Rac-dependent mechanisms and prevented in vivo migration of labeled monocytes to the aorta in response to exogenous tumor necrosis factor-\(\alpha\) and DPP-4.

Conclusion—DPP-4i exerts antiatherosclerotic effects and reduces inflammation via inhibition of monocyte activation/chemotaxis. These findings have important implications for the use of this class of drugs in atherosclerosis. (Circulation. 2011;124:00-00.)

Key Words: adenosine ■ atherosclerosis ■ diabetes mellitus ■ inflammation ■ monocyte chemoattractant proteins ■ nitric oxide

Type II diabetes mellitus is a metabolic disorder with multiple derangements in metabolic and immune pathways that predispose individuals to cardiovascular disease. Although glycemic control is of paramount importance, recent data indicate that broad pleiotropic strategies that target multiple pathways are more likely to succeed in reducing cardiovascular risk in these patients. Emerging evidence suggests an important role for the incretin hormone glucagon-like peptide (GLP-1) in the regulation of postprandial glycemic control and satiety. GLP-1 is inactivated by the exopeptidase dipeptidyl-peptidase 4 (DPP-4), and both inhibitors of DPP-4 (DPP-4i) and DPP-4-resistant incretin analogs are widely used treatment strategies in type II diabetes mellitus. DPP-4 is expressed in inflammatory cells, including monocyte/macrophages, and was originally described as a T-cell differentiation/activation marker. DPP-4 is a high-affinity ligand for membrane-bound adenosine deaminase and greatly potentiates the activity of adenosine deaminase to inactivate adenosine to inosine. These data suggest that DPP-4 may affect leukocyte migration via the modulation of pericellular purinergic stimuli. In this study, we investigated the effects of DPP-4i in a model of atherosclerosis/insulin resistance.
Methods

All experiments were performed in accordance with University Laboratory Animal Accreditation Committee guidelines at Ohio State University.

Animal Models

Male low-density lipoprotein receptor-deficient (LDLR−/−) mice (n=48; 6 weeks old; Jackson Laboratory, Bar Harbor, ME) were allowed to acclimatize for 4 weeks. The mice were then placed on a high-fat diet (60% energy from fat, Research Diets D12492) or standard laboratory diet (Harlan Teklad, Madison, WI). After 4 weeks on a high-fat diet, baseline metabolic parameters like fasting glucose, insulin, oral glucose tolerance test, weights, and blood pressure were measured. Each group was assigned to treatment with alogliptin (40 mg·kg−1·d−1; Takeda Pharmaceuticals, Oak Grove, IL) or a vehicle for 12 weeks (Figure 1). Alogliptin (2-({6-[(3R)-3-aminopiperidinyl-1-yl]-3-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl}[methyl]benzonitrile monobenzoate) is a selective DPP-4i, with prior studies demonstrating specific effects on DPP-4 with improvement in glycemic control.18

The dose for alogliptin was estimated from prior studies in diabetic models.18 Food intake, water intake, weight, and blood pressure were measured weekly, and the alogliptin dose was adjusted accordingly to maintain a constant daily dose. Systolic blood pressure was measured weekly in conscious mice with a computerized noninvasive tail-cuff manometry system (Visitec IFTC model 129 system, Visitech Systems, Apex, NC). Mice were trained for 2 weeks before the experimental procedure.

Systolic blood pressure was recorded for 14 weeks (2 weeks before the start of treatment and 12 weeks of treatment; n=7 per group). The first 10 of 20 systolic blood pressure values recorded were disregarded, and the remaining 10 values were collected for analysis. In additional experiments conducted in apolipoprotein E−deficient (ApoE−/−) mice (n=15; 15 weeks of age; Jackson Laboratory, Bar Harbor, ME), animals were allowed to acclimatize for 1 week before experimentation. The mice were placed on a high-fat diet (40% energy from fat; Harlan Diet, Indianapolis, IN) for a duration of 2 weeks, after which they were assigned to treatment with sitagliptin (MK-0431) mixed in water (80 mg·kg−1·d−1; Merck Pharmaceuticals, Whitehouse Station, NJ) or vehicle for an additional 2-weeks. After 2 weeks, mice were euthanized 16 hours after injection of labeled bone marrow–derived macrophages, as described below.

Metabolic Assessment, Plasma DPP-4 Activity, and Cytokine Analysis

A detailed description of the methods used is available in the online-only Data Supplement.

Visceral Adipose Assessment and Quantification of Visceral Fat by Magnetic Resonance Imaging

A detailed description of the methods used is available in the online-only Data Supplement.

Flow Cytometry and Fluorescence-Activated Cell Sorter Analysis

 Epididymal fat and aorta were isolated from euthanized mice and minced in PBS. Minced tissues were incubated with Collagenase II (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 minutes in a shaker.
The digested tissue was centrifuged to obtain stromal vascular fraction pellet, which was further filtered through a cell strainer. Viable leukocytes were obtained from both aorta and epididymal fat with Lympholyte M (Cedarlane Laboratories Ltd, Burlington, NC).

Flow Cytometry
Leukocytes were isolated from aorta and visceral fat and suspended in PBS containing 5% BSA and 0.02% sodium azide (flow buffer). Cells were washed twice and incubated with anti-CD11b, anti-CD11c, anti Ly6C<sup>+</sup>, anti-CD11b, and CD206 antibodies for 30 minutes. Cells were subsequently washed with flow buffer, resuspended in 1% formalin, and analyzed with a BD fluorescence-activated cell sorter Diva software (Becton Dickinson, San Jose, CA) and BD fluorescence-activated cell sorter Diva software (Becton Dickinson).

Quantification of Atherosclerosis and Functional Vascular Assessment
Detailed methods are provided in the online-only Data Supplement. The heart and ascending aorta were dissected and fixed with paraformaldehyde for 72 hours. The aortic root and adjacent heart were embedded in paraffin, and 5-µm-thick sections were obtained from the annulus extending through the sinus region. Sections were stained with hematoxylin and eosin, Masson trichrome, and Oil Red O. Atherosclerotic quantification was performed as described previously.<sup>19</sup> Immunofluorescent staining for quantification of F<sup>4/80</sup>-positive cells was performed as previously described.<sup>20,21</sup> Thoracic aortic ring segments were suspended in myograph chambers and evaluated as previously described.<sup>21</sup>

Quantitative Real-Time Polymerase Chain Reaction of Gene Expression in CD11b<sup>+</sup> Cells in Aorta and Adipose Stromal Vascular Fraction
Real-time polymerase chain reaction was performed with RNA extracted from CD11b<sup>+</sup> cells from bone marrow and stromal vascular fraction obtained from visceral adipose tissue as detailed in the online-only Data Supplement. Primers used in the work are detailed in the online-only Data Supplement and were designed with the use of National Center for Biotechnology Information resources.

In-Vivo Migration Assays in Atherosclerosis
A detailed methods description is available in the online-only Data Supplement.

In Vitro Monocyte Migration Assays
Human monocytes (THP-1) and mouse bone marrow–derived monocytes were used to study migration patterns with DPP-4i agents (50 µmol/L alogliptin and 100 µmol/L sitagliptin) as described in the online-only Data Supplement. We based our in vitro doses on human pharmacokinetics studies in which levels such as those used may be attained after oral dosing of the drug.<sup>22–24</sup>

Rac-1 Activity
Total Rac1 and activated Rac1 (GTP bound) were quantified from monocytes with Western blot analysis. Half of the cell lysates were subjected to a commercially available Rac1/Cdc42 activation assay kit (Upstate Biotechnology, Temecula, CA), which precipitated activated Rac-1. All samples were boiled in Laemmli buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) and then subjected to SDS-PAGE (12% acrylamide). Rac-1 was quantified with Rac-1 antibody (Millipore, Temecula, CA).

Filamentous Actin Staining
A detailed methods description is available in the online-only Data Supplement.

Statistical Analysis
Data are expressed as mean±SD unless otherwise indicated. Comparison of continuous variables was conducted with 2-way ANOVA to account for the effect from both treatment and diet. If significant results were identified in 2-way ANOVA, post hoc Bonferroni correction was applied for multiple comparison adjustment. In all tests, <i>P</i>&lt;0.05 was considered significant. For responses measured repeatedly at different time points, the test was applied to the measurements at the last time point to determine the overall effect. Statistical analysis was performed with Graph Pad Prism (version 4).

Results
Long-Term DPP-4 Inhibition Improves Metabolic Indexes and Blood Pressure
The nomenclature used for the 4 different groups in the entire article is as follows: normal chow diet (N), vehicle control (V), high-fat diet (H), and drug treatment with a DPP-4i (alogliptin; D). Baseline glucose and post–glucose-load tolerance tests were significantly different between the N and D groups after 4 weeks of high-fat diet feeding (Figure I in the online-only Data Supplement). High-fat diet–fed LDLR<sup>−/−</sup>
mice gained significantly more weight compared with normal chow diet–fed mice at the end of 12 weeks. Table 1 depicts weights, food intake, water intake, and lipid and metabolic parameters at the end of the 12-week period. DPP-4i did not change weekly weight or food consumption in either diet group. After 12 weeks, the HD group exhibited significantly lower levels of fasting and post–glucose-load levels compared with the HV group (Figure 1B and Table 1). Figure 1C demonstrates a marked improvement in insulin resistance (homeostasis model assessment–insulin resistance) and β-cell function (homeostasis model assessment–β) in HD mice compared with the HV group. Systolic blood pressure values increased over time in response to the high-fat diet, with lowering of systolic blood pressure in the HD group, an effect that was apparent as early as 4 weeks after treatment (Figure 1D). Administration of DPP-4i reduced DPP-4 activity in the plasma, as expected, in both ND and HD mice. High-fat diet increased DPP-4 activity in the plasma by 2.9-fold in HV compared with the NV group (Figure 1E). Plasma GLP-1 levels were significantly elevated in response to DPP-4i in the ND and HD groups. Figure II in the online-only Data Supplement depicts plasma cytokine and chemokine measures. The HV group demonstrated an increase in plasma tumor necrosis factor-α (TNFα) compared with NV, whereas DPP-4i resulted in a reduction of TNFα. Interleukin-6 levels were reduced in the HD group compared with the HV group.

### DPP-4 Inhibition Results in Visceral Adipose Remodeling

Figure 2A depicts quantification of visceral and subcutaneous adipose tissue in mice from various groups. HV mice had a 2.8-fold increase in abdominal adipose deposition that was attenuated in the DPP-4i group compared with vehicle. These changes were accompanied by smaller adipocyte size/area in the drug group compared with vehicle (Figure 2B). Figure 2C illustrates a reduction in plasma leptin and resistin levels in the DPP-4i group compared with the vehicle-treated HV animals. Plasma adiponectin levels were reduced in response to high-fat diet with a reversal of this decrease with DPP-4i.

### Long-Term DPP-4i Reduces Recruitment of Inflammatory Monocyte/Macrophages

To assess the effects of DPP-4i on adipose inflammation, we analyzed adipose tissue macrophage content in the stromal vascular fraction and analyzed gene expression for classic markers of M1 and M2 differentiation. Immunohistochemical analysis of visceral adipose samples demonstrated a reduction in F4/80+ macrophages with DPP-4i (Figure 3A). Figure 3B depicts flow cytometric studies on the stromal vascular fraction extracted from the adipose tissue. As is evident in Figure 3B, there was a 315% increase in CD11b+Ly6C+ cells with high-fat diet compared with normal chow diet when adjusted for weight of adipose tissue. DPP-4i reduced CD11b+Ly6C+ cells by 100%. These results sug-

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Figure 2. Effects of dipeptidyl-peptidase 4 (DPP-4) inhibition on visceral adipose measures. A, T1-weighted spin-echo images and analysis of fat mass distribution in all 4 groups. B, Hematoxylin and eosin–stained sections of epididymal fat pads. The box plot represents the upper and lower quartiles. C, Plasma levels of adiponectin, resistin, and leptin in experimental groups. Values are mean±SD. *P<0.05 vs normal chow (N)–vehicle (V), †P<0.05 vs NV, and ‡P<0.05 vs high-fat diet (H)–vehicle in all experiments; n=10 animals per group. D indicates drug treatment.
Figure 3. Effects of long-term dipeptidyl-peptidase 4 (DPP-4) inhibition on adipose tissue macrophages (ATMs). A, Immunofluorescence localization of ATMs (F4/80) in epididymal fat and their quantification. Adipocytes identified by caveolin and nuclei labeled with DAPI. Scale bar=100 μm. F4/80+ nuclei were counted and expressed as a percentage of the total nuclei/×400 power field. Four sections were evaluated from each group. B, Representative flow cytometric dot plots showing double-positive ATMs from mouse epididymal fat. The monocyte macrophage marker CD11b+ (y axis) is shown vs inflammatory CD11c+Ly6C+ cells. C, Gene expression by real-time polymerase chain reaction of “prototypical” M1 and M2 markers in stromal vascular fraction extracted from epididymal fat. Values are mean±SD. *P<0.01 vs normal chow (N)–vehicle (V), †P<0.01 vs NV, and ‡P<0.01 vs high-fat diet (H)–vehicle. n=7 animals per group in all experiments. D indicates drug treatment.
gest that DPP-4i treatment may modulate recruitment of proinflammatory monocytes.25,26 Prior work by Lumeng and colleagues27 has demonstrated an important role for proinflammatory CD11b⁺CD11c⁺ adipose tissue macrophages in the pathogenesis of insulin resistance. DPP-4i treatment decreased double-positive cells by 200% compared with HV mice.

We then analyzed the expression of genes that typify M1 (classically activated) and M2 (alternatively activated) macrophage activation states using the stromal vascular fraction of experimental mice. Recent studies seem to indicate excessive activation of toll-like receptor-4 pathways by endogenous toll-like receptor-4 ligands such as free fatty acids in insulin resistance, with an effect of DPP-4i on toll-like receptor-4 signaling.28,29 Figure 3C depicts an increase in the expression of M2 markers (CD163 and Ym-1) with DPP-4i treatment without changes in other M1 markers, including TNFα and interleukin-6. Taken together, these results suggest DPP-4i reduces proinflammatory gene expression and mediates reversion to an alternatively activated macrophage phenotype.

**Long-Term DPP-4 Inhibition Results in Reduced Plaque Burden and Inflammation**

Figure 4A depicts the results of quantitative evaluation of plaque burden at the level of the aortic sinuses. The HV group was associated with a 6-fold increase in plaque burden compared with the NV group, whereas DPP-4i reduced plaque by 3.5-fold compared with HV. This reduction was associated with a parallel decline in plaque macrophages (Figure 4B) and plaque collagen (Figure 4C). Figure 4D depicts flow cytometric evaluation of CD11b⁺ and CD206⁺ macrophages within plaque. Overall CD11b⁺ cells constituted 30% of the overall mononuclear population in the aorta of the NV group and 50% of the cell population in the HD group. The HV group revealed a 3-fold increase in the number of CD11b⁺ macrophages and a 5-fold increase in CD11b⁺CD206⁺ macrophages. There was a reduction in CD11b⁺ and CD11b⁺CD206⁺ cells in the HD groups compared with HV. Figure III in the online-only Data Supplement illustrates the results of myograph experiments performed on thoracic aortic segments. DPP-4i resulted in an improvement in acetylcholine-dependent relaxation of aortic segments and an attenuation of vasoconstriction in response to phenylephrine. Table 2 depicts the EC₅₀/ED₅₀ values and percent maximal response to the various interventions.

**Effects of DPP-4 Inhibition on Activation and Migration of Immune Cells**

To evaluate the effects of DPP-4i on monocyte gene expression, we analyzed expression of candidate inflammatory pathways. CD11b⁺ cells were isolated from bone marrow by fluorescence-activated cell sorter from the various experimental groups. Treatment with a DPP-4i resulted in reduction in TNFα (P < 0.05); a decreasing trend, although not statistically significant, was also seen in interleukin-6 (P = 0.06) and monocyte chemoattractant protein-1 (P = 0.06) mRNA levels, suggesting an effect on the inflammatory potential of these cells (Figure 5A). CD11b monocytes from HV animals highly expressed DPP-4 compared with normal chow diet–fed animals. GLP-1R was also expressed by these cells with no difference between experimental groups (data not shown).

In vitro transmigration assays on cultured human and murine bone marrow–derived monocytes were performed to explore the effects of DPP-4i on the migratory properties of these cells. Migration assays were performed in response to CCL-2 and CCL-5 gradients in the presence and absence of nanomolar TNFα as described previously.30,31 DPP-4 is known to associate with adenosine deaminase and may thereby modulate pericellular adenosine concentrations.11,14 Experiments were performed in the presence and absence of adenosine receptor antagonists (Ad2RA) and an inhibitor of Rac activation. Figure 5B shows responses in cultured murine bone marrow–derived monocytes; Figure IVA in the online-only Data Supplement depicts responses to CCL-5, and supplement Figure IVB in the online-only Data Supplement illustrates parallel experiments performed on human bone marrow–derived monocytes (THP-1 cells). TNFα stimulation
potentiates monocyte migration to a CCL-2 gradient. Inhibition of DPP-4 with both alogliptin and sitagliptin reduced monocyte migration in response to CCL-2. Preincubation of monocytes with adenosine receptor 2A antagonist (ZM 241385) resulted in the neutralization of the antichemotactic effect of alogliptin (Figure 5C). Preincubation with Exendin(9–39) (an inactive nonmammalian GLP-1 fragment that antagonizes GLP-1) had no effect on monocyte migration in response to the stimuli. Pretreatment with the Rac1 inhibitor NSC 23766 resulted in a decreased migration to TNFα stimulation, indicating that Rac1 is involved in chemotaxis. Parallel Rac-GTP pull-down assays were performed to detect the effects of alogliptin and sitagliptin pretreatment on the activation of Rac and to evaluate the role of adenosine 2A receptor in modulating the effects of these drugs (Figure 5C and Figure V in the online-only Data Supplement). Pretreatment with the DPP-4i alogliptin (50 μmol/L) and sitagliptin (100 μmol/L) reduced the level of active Rac1 after stimulation by TNFα and monocyte chemotactic protein-1. This effect was blunted by adenosine receptor 2A antagonist (ZM 241385) after pretreatment with alogliptin (Figure 5C) but not by sitagliptin (Figure V in the online-only Data Supplement). The reasons for the difference between the 2 compounds on adenosine receptor signaling are currently unclear but may relate to a combination of chemical structure differences, dose, or both. Figure 5D shows actin polymerization induced by TNFα that was inhibited with the pretreatment of cells with alogliptin. Additionally, antipolymerization effects on actin filaments by alogliptin were reversed by adenosine receptor 2A antagonist (ZM 241385). These experiments suggest that DPP-4i results in decreased chemotaxis of immune cells via Rac1-dependent pathways.

To further evaluate the significance of the in vitro effects of DPP-4i on migration and to validate the effects in an alternate model of atherosclerosis, we conducted experiments on ApoE−/− mice on high-fat diet that were treated with sitagliptin or vehicle for a 2-week period. Two weeks of treatment with sitagliptin reduced DPP-4 activity by >75% (data not shown). We used an in vivo model of chemotactic migration to plaque using an inflammatory stimulus (TNFα injected intraperitoneally). The injection of labeled cells (carboxyfluorescein succinimidyl ester) has previously been shown to migrate to the plaque, with the extent of migration representing an index of chemotactic ability. We first confirmed that these cells indeed express DPP-4. Figure 6A depicts results of flow cytometry that reveal that >90% of these cells express DPP-4. Figure 6B represents flow cytometric plots for carboxyfluorescein succinimidyl ester–labeled bone marrow–derived monocytes isolated from the aorta of ApoE−/− mice. We then evaluated the effects of exogenously injected DPP-4. The administration of DPP-4 in these experiments revealed a marked promigratory effect of this protein consistent with prior experiments in lymphocytes. Pretreatment with DPP-4i prevented the migration of cells to the aorta (Figure 6C). Figure V in the online-only Data Supplement depicts results of migration to the peritoneal cavity under identical conditions performed in a subset of mice that demonstrated a potent effect of DPP-4i in preventing migration into the peritoneal cavity in response to DPP-4 and TNFα.

**Discussion**

In this article, we demonstrate an important effect of DPP-4i in reducing atherosclerosis. Reduction in atherosclerosis was accompanied by favorable alterations in metabolic and inflammatory parameters. Notably, these changes occurred without differences in weight loss in the animals, consistent with weight neutrality of DPP-4i noted in clinical studies. The mechanisms appear to be related to inhibition of DPP-4–mediated alterations in monocyte chemotaxis, occurring via changes in Rac-dependent actin–cytoskeletal interactions.

**Table 2. Physiological Responses in Isolated Aortic Ring Segments From the Treatment Groups**

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<td>98±8.8</td>
<td>186±13.2*</td>
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<td>−7.3 to −6.4</td>
<td>−6.9 to −6.2</td>
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<td>−90±6.4†</td>
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*P<0.05 vs normal chow diet—vehicle.
†P<0.05 versus high-fat diet—vehicle; n=5 animals per group.
Figure 5. Effects of dipeptidyl-peptidase 4 (DPP-4) inhibition (DPP-4i) on bone marrow–derived monocyte gene expression and migration.

A, Gene expression in CD11b+ cells isolated from the bone marrow after 12 weeks of treatment with DPP-4i (D) or vehicle (V) in normal chow diet (N) and high-fat diet (H) groups; n=5 to 7 animals per group. *P<0.05 vs NV.

B, Migration assays index in response to various interventions assessed by Boyden chamber. The white and black bars represent migration assays done with alogliptin and sitagliptin as DPP-4i, respectively. 1=control; 2=CCL-2; 3=CCL-2+pretreatment with either sitagliptin or alogliptin; 4=tumor necrosis factor-α (TNFα)+CCL-2; 5=TNFα+CCL-2+pretreatment with NSC23766; 6=TNFα+CCL-2+pretreatment with both DPP-4i agents; 7=TNFα+CCL-2+pretreatment with DPP-4i agents and ZM241385; 8=TNFα+CCL-2+pretreatment with DPP-4i agents and Exendin(9–39); 9=TNFα+CCL-2+pretreatment with Exendin(9–39) amide only.

C, GTP-bound Rac (active) estimated with a pull-down assay with various interventions. D, Confocal microscopy after phalloidin staining in response to various interventions. Values are mean±SD; *P<0.05 vs CCL-2 stimulation only (lane 2) and #P<0.05 vs CCL-2+TNFα stimulation (lane 4). n=3 independent experiments. MCP-1 indicates monocyte chemoattractant protein-1; IL, interleukin; IFN, interferon; and iNOS, inducible nitric oxide synthase.
peptide, thereby influencing glycemic control.\textsuperscript{36,37} However, the nonglycemic role of DPP-4 and, in particular, its role in the regulation of inflammation may allow its participation in the pathogenesis of chronic inflammatory disease.

DPP-4i resulted in improvements in insulin resistance, postprandial glucose, inflammatory cytokines, and adipokines in the plasma. A reduction in inflammatory monocytes (CD11b$^+$Ly6C$^{hi}$) was discernible in the adipose, along with improvement in adipocyte area and a reduction in visceral adipose content. There was a reduction in monocytes in the plaque-expressing proinflammatory genes. These changes suggest potent effects of DPP-4i on inflammation, medi-
ated potentially via its effects on monocyte recruitment to tissue niches and alteration of monocyte activation, as evidenced by an increase in markers of alternate macrophage activation such as CD163 and Ym-1 in adipose tissue macrophages.

How might DPP-4i modulate monocyte recruitment into visceral adipose tissue? DPP-4 exopeptidase activity has been shown to play a role in the processing of chemokines and may modulate the chemotactic behavior of cells.26,28,29 The enzyme has been described as an adenosine deaminase–binding protein.11–14 Membrane-bound DPP-4 is a high-affinity receptor for adenosine deaminase. Binding to adenosine deaminase does not require the catalytic domain of DPP-4 and does not induce enzymatic or conformational changes. This pair of enzymes is thought to be involved in immunoregulatory mechanisms through the control of pericellular adenosine concentrations.11,12 Recent studies indicate that purinergic stimuli are critical in mediating movement of inflammatory cells toward a chemokine stimulus.15,30 Activation of adenosine A2B receptors has been shown to dampen chemotaxis and chemokinesis.30 Results from migration assays seem to suggest a role for DPP-4 in regulating chemotaxis in response to chemokines (CCL-2 and CCL-5) and inflammatory mediators (TNFα) that is dependent on Rac signaling. Indeed, monocytes upregulated DPP-4 expression both in vivo and in response to differentiation in vitro. High-fat feeding markedly increased DPP-4 activity and expression. With these results taken together with the promigratory properties of this molecule, it is reasonable to hypothesize that the effects of DPP-4i may be modulated through effects on this pathway.

Active, GTP-bound Rac1 plays an important role in regulating cell migration through the modulation of actin-rich lamellipodial protrusions, critical components for generating the driving force of cell movement. In several systems, inhibition of Rac results in cessation of cell movement.31,32,33 In our study, preincubation of monocytes with a Rac-1 antagonist resulted in the neutralization of the antichemotactic effect of alogliptin and sitagliptin, whereas Exendin(9–36) (inactive GLP-1 fragment) did not have such an effect, suggesting that the effects on in vitro migration were GLP-1 receptor independent. DPP-4i reduced atherosclerosis and improved vascular function. Both GLP-1 (via DPP-4i) and non–GLP-1 mechanisms may activate PI3K-Akt–endothelial nitric oxide synthase, may improve nitric oxide release, and may confer additional benefits.32,33 Consistent with this, there was a 20-mm Hg reduction in blood pressure at the end of 10 weeks, which was evident as early as 4 weeks after treatment. It is conceivable that the improvement in vascular function and atherosclerosis may have also resulted from the antiinflammatory effects of DPP-4i. Our experiments suggest a prominent effect on inflammatory migration of monocytes as early as 2 weeks after treatment, a time point at which no effects on blood pressure were seen (Figure 1). This finding suggests that the antiinflammatory effects may well precede blood pressure effects and may mediate improvements in endothelial function and atherosclerosis. Nevertheless, it may be difficult to completely exclude the incretin effects of DPP-4i as a potential mediator of beneficial effects in vivo.

The incretin axis (GLP-1 receptors, glucose-dependent insulinotropic polypeptide receptors and DPP-4) is widely expressed in the cardiovascular system, with prior work suggesting that ligation of the GLP-1 receptor by GLP-1 in both cardiomyocytes and endothelial cells resulting in upregulation of cell survival pathways and endothelial function.34–36 Thus, an increase in GLP-1 as a consequence of DPP-4i may represent an important additional mechanism of benefit.37–39 Additional lines of evidence suggest GLP-1–dependent vascular effects of GLP-1 and truncated peptides of GLP-1 such as Exendin(9–36).40 The vasodilatory effects of both GLP-1 and GLP-1(9–36) correlate with an increase in cGMP release and are typically attenuated by preincubation of vessels with nitric oxide synthase inhibitors, suggesting that at least part of their vasodilatory mechanism is nitric oxide/cGMP dependent.

Our results suggest broad pleiotropic effects of DPP-4i and suggest a potential role for these agents in retarding atherosclerosis and related complications in type II diabetes mellitus.

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Disclosures
This study was supported in part by an educational grant from Takeda Pharmaceuticals North America to Dr Rajagopalan. Alogliptin was provided by Takeda Pharmaceuticals. The other authors report no conflicts.

References
DPP-4 Inhibition Reduces Atherosclerosis


27. Shah et al. DPP-4 Inhibition Reduces Atherosclerosis

11


CLINICAL PERSPECTIVE

The incretin hormones glucagon-like peptide and glucose-dependent insulotropic polypeptide play a key role in the regulation of postprandial glycemia and satiety. Incretin hormones are inactivated by the exopeptidase dipeptidyl-peptidase 4 (DPP-4). Both small-molecule inhibitors of DPP-4 and DPP-4–resistant incretin analogs are increasingly common treatments for type II diabetes mellitus, although their effects in reducing long-term cardiovascular complications remain to be established. An expanding list of potential beneficial effects of DPP-4 inhibition on the cardiovascular system includes glucagon-like peptide–mediated effects on cardioprotective pathways, nitric oxide–dependent vasodilation, and non–glucagon-like peptide effects that relate to a pathophysiological role for DPP-4 in regulating inflammation. In this study, we investigated the net effects of long-term DPP-4 inhibition with alogliptin in a model of atherosclerosis and insulin resistance. DPP-4 activity was increased in atherosclerosis with a reduction in response to treatment. DPP-4 inhibition improved insulin resistance, blood pressure, and visceral adiposity with reductions in atherosclerosis and inflammation (evidenced by a reduction in plaque and adipose inflammatory macrophage content) and a shift to an alternately activated macrophage phenotype. DPP-4 inhibition prevented monocyte migration and actin polymerization in vitro via Rac-dependent mechanisms and prevented in vivo migration of labeled monocytes to the aorta in response to exogenously administered tumor necrosis factor-α and DPP-4. These data support a net effect of DPP-4 inhibition in reducing adipose and vascular inflammation with a concomitant reduction in atherosclerosis and support a therapeutic role for these agents in preventing cardiovascular complications in type II diabetes mellitus.
SUPPLEMENTAL MATERIAL

Supplemental Methods

Metabolic Assessment, Plasma DPP-4 Activity and Cytokine Analysis

This was performed before the initiation of the DPP-4i treatment and after completion of 12 weeks of treatment (6 animals/group).

Glucose tolerance tests (GTT): Briefly, mice were fasted overnight and their fasting blood-glucose concentration was determined with an Elite Glucometer (Bayer, Mishawaka, IN). Then, the mice were administered an intra-peritoneal dose of glucose (2 mg/g body weight); blood-glucose concentration were measured at 30, 60, 90, and 120 minutes after glucose administration. Blood was collected from mice via retro-orbital puncture after anesthetizing them with isoflurane/O₂ mixture. Mice were then euthanized by cervical dislocation and various organs were harvested. The blood was immediately centrifuged at 4°C and the separated plasma snap frozen and stored at -80°C until further procession. ELISA kits were used to measure total plasma insulin (Crystal Chem Inc., Downers Grove, IL), leptin (Crystal Chem Inc., Downers Grove, IL), adiponectin/acrp30 (R&D Systems, Minneapolis, MN), resistin (R&D Systems, Minneapolis, MN), and the active form of Glucagon-Like Peptide-1 (Millipore, St. Charles, MS). The homeostasis model assessment (HOMA) method of insulin resistance (IR) index was then calculated using the standard HOMA IR and HOMA β equations.¹

Cytokine Measurements: Plasma cytokines were measured from the plasma using Mouse Inflammation 6-Plex Kit from BD Bioscience (San Diego, CA) according manufacture instructions.
Assessment of Plasma DPP-4 Activity: 10 µL of plasma sample was mixed with 40 µL assay buffer containing 250 mmol/L Tris–HCl (pH 7.5), 0.25% (wt/vol) bovine serum albumin, and 0.125% (wt/vol) 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) in 96-well plates. Samples were mixed with 50 µl of 1 mM DPP-4 substrate (BPS Bioscience, San Diego, CA) to initiate the reactions. Samples were allowed to incubate on plate shaker at room temperature. Absorbency was measured at 405 nm and monitored at both 20 and 60 min after the reaction initiation. The DPP-4 activity was assessed by the increase in absorbency between 20 and 60 min. The DPP-4 activity of the plasma from Alogliptin-treated mice was compared with that of the vehicle-treated mice, which was defined as 100% with the substrate.

Visceral Adipose Assessment and Quantification of Visceral Fat by MRI

After 12 weeks of DPP-4i, mice were euthanized and epididymal fat was carefully dissected. A portion of this tissue was fixed with 4% paraformaldehyde/PBS. The adipose tissue was then embedded in paraffin, and 5-µm sections were obtained and mounted on a standard glass slide. For the quantification of the adipocyte area we randomly selected 90 adipocytes from 6 mice from each group and analyzed the area using Metamorph™ software (version 7.1.2.0, Metamorph, Downingtown, PA). For immunofluorescent staining of inflammatory macrophages, paraffin sections were blocked in 5% BSA in PBS with 0.3% Triton X-100 (PBST) for 1 hour and then incubated with anti-F4/80 (Abcam Inc. Cambridge, MA) and anti-caveolin (BD Biosciences, Franklin Lakes, NJ) antibodies at 1:100 dilution with gentle rocking at room temperature for 2-3 hours. After washing, fluorophor-conjugated secondary antibodies
were added to the slides and incubated for 60 minutes at room temperature with gentle rocking. The slides were then washed and 95% glycerol was used as a mounting media to place a cover slip. Nuclei were counter stained with Hoechst 33342 (Sigma-Aldrich). Tissue was imaged using an inverted confocal scanning microscope (Zeiss).

Quantification of visceral fat: Magnetic resonance imaging was performed on a 9.4 T Bruker BioSpin system equipped with ParaVision 4.0 software. A spin echo sequence (repetition time 920 ms; echo time 12 ms, in-plane resolution 256x256 μm; 2, 4 averages) was used to acquire 50 transversal, 1 mm thick slices that covered from the top of the kidneys to the hind legs. Fat quantification analysis was performed using OsiriX software (The Osirix Foundation, Geneva, Switzerland). Thresholding technique was applied to all images to separate fat and water signals. Manual correction of the thresholded fat areas was performed and total fat volume was calculated using OsiriX volume calculator plug-in (sum of slices fat area multiplied by the slice thickness) as previously described.3

Functional Vascular Assessment: Mice were euthanized by cervical dislocation. Thoracic aortas were dissected from the animals and immediately immersed in a physiological salt solution buffer (sodium chloride, 130 mEq/L; potassium chloride, 4.7 mEq/L, calcium dichloride, 1.6 mEq/L; magnesium sulfate, 1.17 mEq/L; potassium diphosphate, 1.18 mEq/L; sodium bicarbonate, 14.9 mEq/L; EDTA, 0.026 mEq/L; and glucose, 99.1 mg/dL [5.5 mmol/L]; pH, 7.4) at room temperature. The aortas were then cleaned of adherent fat/connective tissue and were cut into rings of 2 mm to 3 mm length under a microscope. Vessel rings were mounted in a standard 5 ml organ bath (filled with PSS buffer). The bath medium was maintained at 37°C with a pH of 7.4 and
aerated continuously with 95% oxygen and 5% carbon dioxide. Extra care was taken to ensure that the endothelium was not damaged during the whole process of tissue preparation and mounting.

Briefly, the aortic rings were allowed to equilibrate for 90 minutes at a resting tension of 700 mg, with the bath medium changed every 15 minutes. All preparations were contracted with isotonic, high-potassium physiological saline solution (KPSS 120 mmol/L) to achieve maximum tension. Endothelial integrity was assessed with the single addition of acetylcholine (10 µmol/L) which caused relaxation of the aortic segments, sub maximally precontracted with phenylephrine (10-100 nmol/L). The vessels were then washed thoroughly and allowed to equilibrate for 1 hour before beginning experiments with agonists.

In-vivo migration assays in Atherosclerosis

After 2 weeks of treatment with Sitagliptin or vehicle, plasma DPP-4 activity was measured to confirm the effectiveness of the treatment. Bone marrow derived macrophages (BMDM) from wild type C57Bl/6 mice were isolated and cultured for 1 week. DPP-4 expression was confirmed by flow-cytometry at the end of the culture period. BMDM from these animals were than labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) as per the manufacturers instructions (and injected in the mice via intravenous route (5x10^6 cells per mouse). At the same time mice were intraperitoneally injected with PBS, TNFα (1 µg/kg) or soluble-DPP-4 (10 mU). After 16 hours, the mice were euthanized; the aorta was isolated and digested for performance of FACS analysis as described in
previous sections. In some of the mice, the peritoneal cavity was washed with 4 ml of ice-cold PBS. The recovered cells were also analyzed by flow cytometry).

**Migration Assay**

Murine bone marrow derived cells and human THP1 cells were used to study migration assays. Briefly cells were suspended at 1x10^6 cells/ml in Hepes-buffered Hank’s balanced solution, and chemotaxis was assayed in modified bipartite Boyden chemotaxis chambers. CCL-2 (MCP-1) and CCL-5 RANTES (final concentration 25 nM) was added to the bottom compartment, and the cells were placed in the upper compartment and were allowed to migrate through a polycarbonate filter at 37ºC for 90 minutes. Concomitant treatment with nanomolar concentrations of TNFα were used to additionally activate the monocytes, as previously described. Cells were pretreated with the appropriate agonists (TNFα), with or without DPP-4i and antagonists before the migration assay. Filters were fixed and stained, and cells that had migrated through the filter were counted under a high power field. For each group, 11 high power images were used and all experiments were repeated three times. The migration index was calculated based on the ratio of cells that migrated in response to TNFα (pre-incubation) to cells that migrated in the absence of TNFα, as previously described.

**Filamentous Actin Staining**

Mouse RAW cells were cultured on glass coverslips in 6-well. Cells were pre-treated with Alogliptin (1 µM for 1 hour) or control (PBS) and then stimulated with TNFα (1 picomolar) or TNFα in the presence of ZM241385 (10 µM) for 30 min. Coverslips were washed with PBS and fixed in 4% formaldehyde for 10 min followed by permeabilization with Triton X-100 for 5 min at room temperature. After successive washing with PBS,
the coverslips were treated with 165 nM Alexa Fluor 568-phalloidin conjugate (Invitrogen) for 25 min at +4°C. Next, coverslips were washed twice with PBS and incubated with 1 µM DRAQ5 nuclear stain (Biostatus Limited, UK) for 5 min at room temperature followed by mounting on microscope slides with Fluormount mounting media (Southern Biotechnology Associates; Birmingham, AL). Actin polymerization was visualized under Olympus Spectral FV-1000 confocal microscope using 60x oil immersion objective. Krypton laser (568 nm) and HeNe-red laser (633 nm) were used for excitation of Alexa Fluor 568 and DRAQ5, respectively. Images were processed with Olympus FV-10 ASW viewer and Adobe Photoshop.

**Total RNA Extraction and Quantitative RT-PCR**

RT-PCR was performed using RNA extracted from CD11b+ cells from bone marrow and leukocytes obtained from visceral adipose tissue of the experimental mice. An Absolutely RNA MiniPrep kit (Stratagene, La Jolla, CA, USA) was used to extract Total RNA following the manufacturer's instructions. cDNA was reverse transcribed using 500 nanograms of total RNA per manufacturer's instructions (Roche Diagnostics GmbH Mannheim Germany) using random primers. All real-time reactions had the following profile conditions: 1 cycle at 95°C for 10 minutes; 50 cycles at 95°C for 10 seconds, 59°C for 20 seconds, and 72°C for 20 seconds; and 1 cycle at 58°C for 1 minute. Quantitative real-time PCR was performed with a Lightcycler 480 (Roche Applied Sciences) using SYBR Green I Master Mix (Roche Diagnostics GmbH Mannheim, Germany). The relative quantification values for these gene expressions were calculated from the accurate threshold cycle (CT), which is the PCR cycle at which an increase in reporter fluorescence from SYBR green dye can first be detected above a
baseline signal. The CT from housekeeping genes were averaged and subtracted from the CT values for the gene of interest in each well to calculate ΔCT. All target genes were expressed as fold increased compared to the control. Melting/dissociation curves were run on each plate to assure the production of one amplicon of the same melting temperature for each primer set. Real time primers were designed to span genomic introns, thus avoiding amplification of genomic DNA possible present in the RNA samples. “No template,” cDNA negative controls were included for each gene set in all PCR reactions to detect contamination. The primers used were as follows:
### Supplemental Tables

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Supplement Figure 1

Results of glucose tolerance test at the beginning of 12 week treatment. n= 7/group.

Values are mean ± SD.
**Supplement Figure 2**

Plasma levels of cytokines. n=7/group. *** p<0.01 when compared with NV and # p<0.05 when compared with HV. Values are mean ± SD.
Supplement Figure 3

A. Vasorelaxation of pre-constricted aortic rings in response to acetylcholine (Ach). B. Phenylephrine-induced vasoconstriction expressed as % of peak KCl tension. C. Vasorelaxation in response to sodium nitroprusside (SNP). D. Insulin-mediated vasorelaxation in aortic rings pre-constricted with phenylephrine (10^{-7} mol/L). All values are mean ± SD, n= 5/intervention; * p<0.05 compared with NV, and # p<0.05 when compared with HV.
Supplement Figure 4

A. Migration assays in murine bone marrow derived monocytes stimulated with CCL-5.

Bar graphs labels are as follows 1=control, 2=CCL-5, 3=CCL-5+pretreatment with
Alogliptin. B. Effects of DPP-4i in human THP-1 cells. Pretreatment with ZM 241835 resulted in the neutralization of anti-chemotactic effects of Alogliptin. 1=control; 2=CCL-2; 3=CCL-2+pretreatment with Alogliptin; 4=TNFα+CCL-2; 5=TNFα+CCL-2+pretreatment with NSC23766; 6=TNFα+CCL-2+pretreatment with Alogliptin; 7=TNFα+CCL-2+pretreatment with Alogliptin and ZM241385; 8=TNFα+CCL-2+pretreatment with Alogliptin and exendin; 9=TNFα+CCL-2+pretreatment with exendin only; *** p<0.01 when compared with CCL-2 stimulation only and # p<0.05 when compared with CCL-2 and TNFα stimulation. All values are mean ± SD and represent n=3 experiments/intervention. C. GTP-bound Rac (active) estimated with a pull-down assay with various interventions using sitagliptin as DPP-4i. n=3 independent experiments.
Supplement Figure 5

Representative flow-cytometric dot plots show CFSE labeled BMDM extracted from peritoneal cavity of ApoE⁻/⁻ mice using TNFα or sDPP-4 to induce inflammation.
Supplement Figure 6

Effect of chronic DPP-4 inhibition on atherosclerosis. Images of aortic sinus stained with Masson's trichrome from high fat fed mice treated with DPP-4i and vehicle, respectively. n=8/group.
Supplemental References