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A Dipeptidyl Peptidase-4 Inhibitor, Des-Fluoro-Sitagliptin, Improves Endothelial Function and Reduces Atherosclerotic Lesion Formation in Apolipoprotein E–Deficient Mice

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Objectives The aim of this study was to investigate the antiatherogenic effects of the dipeptidyl peptidase-4 inhibitor, des-fluoro-sitagliptin (DFS).

Background The new class of anti–type 2 diabetes drugs, dipeptidyl peptidase-4 inhibitors, improves glucose metabolism by increasing levels of active glucagon-like peptide (GLP)-1.

Methods Endothelial function was examined by acetylcholine-induced endothelium-dependent vasorelaxation using aortic rings and atherosclerotic lesion development in the entire aorta in apolipoprotein E–deficient mice fed a high-fat diet with or without DFS, and the antiatherogenic effects of DFS were investigated in cultured human macrophages and endothelial cells. Plasma levels of active GLP-1 were measured in patients with or without coronary artery disease.

Results DFS significantly improved endothelial dysfunction (89.9 $\pm$ 3.9% vs. 79.2 $\pm$ 4.3% relaxation at 10^{-4} mol/l acetylcholine, $p < 0.05$) associated with increased endothelial nitric oxide synthase phosphorylation and reduced atherosclerotic lesion area (17.7% [15.6% to 25.8%] vs. 24.6% [19.3% to 34.6%], $p < 0.01$) compared with vehicle treatment. In cultured human macrophages, DFS significantly increased GLP-1-induced cytosolic levels of cyclic adenosine monophosphate compared with GLP-1 alone, resulted in inhibiting phosphorylation of c-jun N-terminal kinase and extracellular signal-regulated kinase 1/2 and nuclear factor-kappa B p65 nuclear translocation through the cyclic adenosine monophosphate/protein kinase A pathway, and suppressed proinflammatory cytokines (i.e., interleukin-1-beta, interleukin-6, and tumor necrosis factor-alpha) and monocyte chemoattractant protein-1 production in response to lipopolysaccharide. DFS-enhanced GLP-1 activity sustained endothelial nitric oxide synthase phosphorylation and decreased endothelial senescence and apoptosis compared with GLP-1 alone. In the human study, fasting levels of active GLP-1 were significantly lower in patients with coronary artery disease than those without (3.10 pmol/l [2.40 to 3.62 pmol/l] vs. 4.00 pmol/l [3.10 to 5.90 pmol/l], $p < 0.001$).

Conclusions A DPP-4 inhibitor, DFS, exhibited antiatherogenic effects through augmenting GLP-1 activity in macrophages and endothelium. (J Am Coll Cardiol 2012;59:265–76) © 2012 by the American College of Cardiology Foundation

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Abbreviations and Acronyms

apoE = apolipoprotein E

CAD = coronary artery disease

cAMP = cyclic adenosine monophosphate

DFS = des-fluoro-sitagliptin

DM = diabetes mellitus

DPP = dipeptidyl peptidase

eNOS = endothelial nitric oxide synthase

ERK = extracellular signal-regulated kinase

GLP = glucagon-like peptide

HCAEC = human coronary artery endothelial cell

HFD = high-fat diet

IL = interleukin

JNK = c-jun N-terminal kinase

LPS = lipopolysaccharide

mRNA = messenger ribonucleic acid

NF-κB = nuclear factor–kappa B

PKA = protein kinase A

Diabetes mellitus (DM) increases the risk for atherosclerosis and cardiovascular disease, but whether antidiabetic medications reduce cardiac events remains controversial (1). Moreover, intensive glucose control does not seem to be beneficial in preventing major cardiovascular events compared with standard therapy (2). The new class of anti–type 2 DM drugs, dipeptidyl peptidase (DPP)-4 inhibitors and glucagon-like peptide (GLP)-1 analogues, improve glucose metabolism through the activation of GLP-1 receptor signaling, which induces insulin secretion and suppresses glucagon secretion in the pancreas (3). Several studies have shown the beneficial effects of GLP-1 on the cardiovascular system (4,5). In contrast, there is a lack of evidence for the beneficial effects of DPP-4 inhibitors on the cardiovascular system.

Atherosclerosis is an inflammatory disease of the arterial wall, and endothelial dysfunction has been shown to contribute to atherogenesis (6,7). Approaches designed to reduce the inflammatory activity and improve endothelial function may have additional therapeutic value in the prevention and treatment of atherosclerotic diseases, including coronary artery disease (CAD) (8).

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In the present study, we tested the hypothesis that enhancement of GLP-1 action with a DPP-4 inhibitor has anti-inflammatory effects on macrophages and can result in amelioration of endothelial dysfunction, leading to a slowdown in the progression of atherosclerosis. For this purpose, we investigated the antiatherogenic effects of a DPP-4 inhibitor in apolipoprotein E (apoE)–deficient mice fed a high-fat diet (HFD) with or without a DPP-4 inhibitor. In a human study, we also measured fasting plasma levels of active GLP-1 in patients with or without CAD.

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Methods

The DPP-4 inhibitor, des-fluoro-sitagliptin (DFS) was supplied by Merck & Company, Inc. (Rahway, New Jersey). In animal experiments, 4-weeks-old apoE-deficient mice (C57BL/6.KOR-Apoeshl) were purchased from SLC, Inc. (Shizuoka, Japan). The mice were fed a standard diet (Research Diets, New Brunswick, New Jersey) for 2 weeks, followed by an HFD (35% kcal from fat; Research Diets). For mice in the DFS group, DFS was mixed with baked HFD at a final concentration of 0.3% (corresponding to 200 mg/kg/day). After treatment for 16 weeks, whole aortas were collected and stained with Oil Red O. The extent of atherosclerosis was expressed as the percent of the lesion area extending from the ascending aorta to the abdominal bifurcation.

In cell culture studies, the human monocytic cell line THP-1 was purchased from the American Type Culture Collection (Manassas, Virginia). These cells were cultured additionally in 10 ng/ml granulocyte macrophage colony-stimulating factor, 10 ng/ml macrophage colony-stimulating factor, and 16 nmol/l phorbol 12-myristate 13-acetate for differentiation into macrophages. These cells were then used as "THP-1 cell-derived macrophages" after the 4-days culture. Human coronary artery endothelial cells (HCAECs) (Lonza Walkersville, Inc., Walkersville, Maryland) and human aortic endothelial cells (Lonza Walkersville, Inc.) were cultured in EGM-2MV medium (Lonza Walkersville, Inc.) under standard culture condition. Cells were pre-treated with or without DFS (2 μmol/l) for 1 h, followed by treatment with or without GLP-1 (10 pmol/l).

In human studies, plasma concentrations of active GLP-1 were measured in patients admitted to Kumamoto University Hospital (Kumamoto, Japan). A total of 200 patients with or without CAD were recruited.

Statistical analysis. Group data of normally distributed continuous variables are expressed as mean ± SD or mean ± SEM as indicated. Medians and interquartile ranges are reported for parameters with skewed distributions. Differences between 2 variables were tested using unpaired Student's t tests or Mann-Whitney U tests. Data of multiple groups were compared using 1-way analysis of variance followed by post hoc Tukey-Kramer tests. A p value < 0.05 was considered statistically significant. All analyses were performed using SPSS version 17.0J for Windows (SPSS Japan Inc., Tokyo, Japan).

An expanded Methods section is available in the Online Appendix.

Results

Animal experiments. DFS REDUCED ATHEROSCLEROTIC LESION FORMATION. ApoE-deficient mice were treated for 16 weeks with DFS (Fig. 1A). As shown in Online Appendix Table 1, DFS had no significant effects on mean body weight, systolic blood pressure, heart rate, lipid profile, fasting glucose, and insulin. The relative surface area of the atherosclerotic lesion was significantly smaller in the DFS

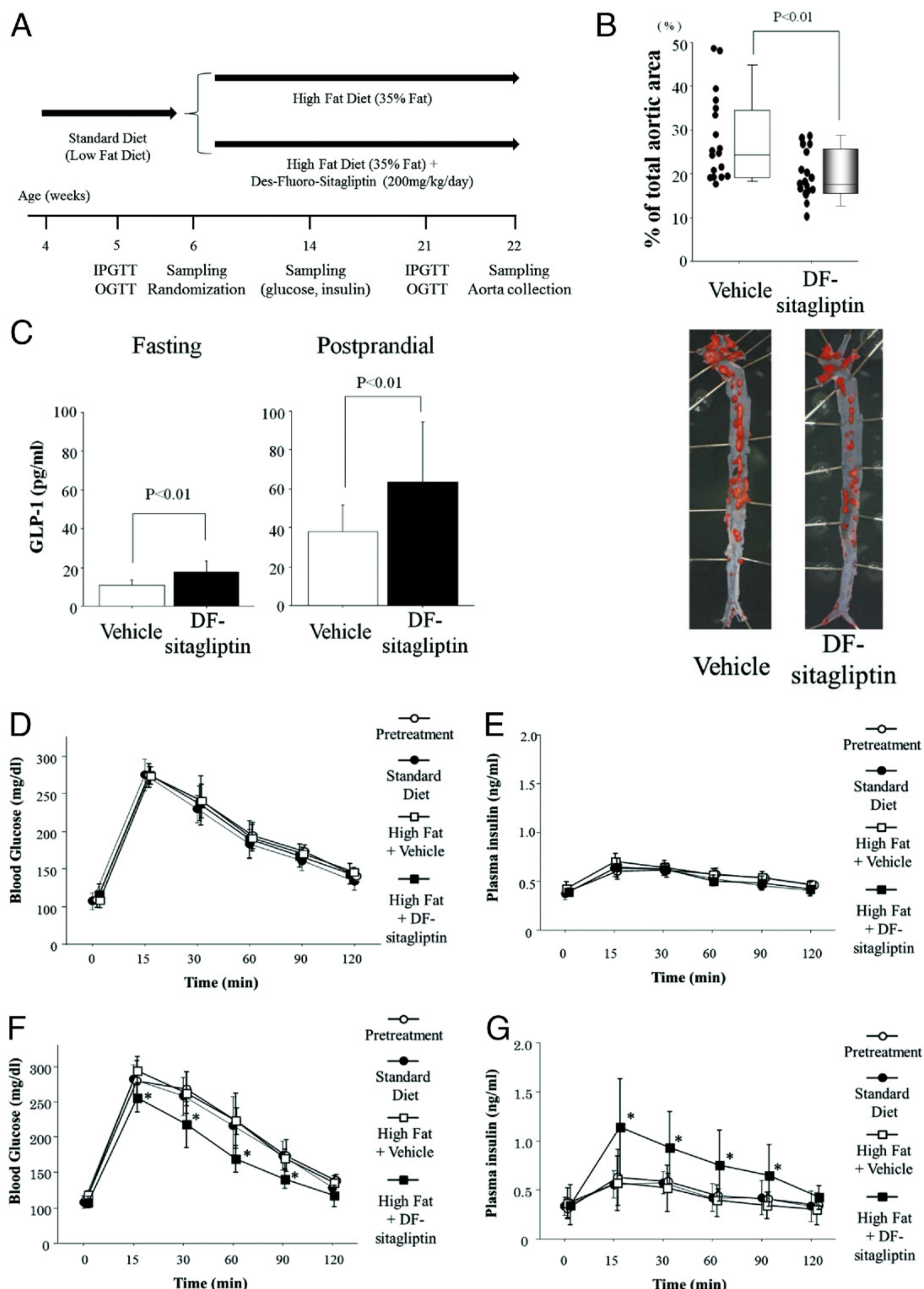

Figure 1 **DFS Reduces Atherosclerotic Lesion Formation in ApoE-Deficient Mice**

(A) The protocol used for analysis of the antiatherogenic effects of des-fluoro-sitagliptin (DFS). **(B)** The relative surface area of the atherosclerotic lesion in vehicle-treated and DFS-treated apolipoprotein E (apoE)–deficient mice (n = 18 each; p values by Mann-Whitney *U* tests). **(C)** Fasting and postprandial plasma levels of active glucagon-like peptide (GLP)-1 in DFS-treated and vehicle-treated apoE-deficient mice (n = 18 each; p values by Student's *t* tests). **(D)** Plasma glucose levels during intraperitoneal glucose tolerance test (IPGTT). **(E)** Plasma insulin levels during IPGTT. **(F)** Plasma glucose levels during oral glucose tolerance test (OGTT). **(G)** Plasma insulin levels during OGTT. Values are mean ± SD.

group compared with the vehicle group (17.7% [15.6% to 25.8%] vs. 24.6% [19.3% to 34.6%], n = 18 each, $p < 0.01$) (Fig. 1B). The fasting and post-prandial plasma levels of active GLP-1 were higher in the DFS group than in the vehicle groups (fasting: 17.8 ± 6.2 pg/ml vs. 10.8 ± 2.9 pg/ml; post-prandial: 63.5 ± 30.8 pg/ml vs. 38.1 ± 13.3 pg/ml; n = 18 each; $p < 0.01$) (Fig. 1C). Furthermore, the univariate logistic regression analysis revealed that only fasting levels of active GLP-1 were significantly correlated with the high percent atherosclerotic area group (odds ratio: 0.80; 95% confidence interval: 0.67 to 0.97; $p < 0.05$), but not the other factors. Intraperitoneal glucose tolerance testing showed no differences in plasma glucose and insulin concentrations between the DFS and vehicle groups or even between the pre-treatment and standard diet treatment groups (Figs. 1D and 1E). On oral glucose tolerance testing, the blood glucose levels after glucose load were significantly decreased and the plasma insulin levels after glucose load were significantly increased in the DFS group than the other groups. However, the glucose and insulin levels at 0 and 120 min after glucose load were not different among all groups (Figs. 1F and 1G).

Histological analysis of Oil Red O–stained sections of the aortic sinus indicated a significant reduction in lipid-rich atherosclerotic lesion area in the DFS group compared with the vehicle group (42.6 ± 3.5% vs. 48.9 ± 6.7%, n = 18 each, $p < 0.01$) (Fig. 2A). Histological analysis of macrophage-stained aortic sinus also indicated that DFS treatment significantly reduced the area of macrophages in plaques compared with vehicle treatment (15.6 ± 6.9% vs. 20.8 ± 6.7%, n = 18 each, $p < 0.05$) (Fig. 2B). Furthermore, DFS treatment significantly decreased T lymphocyte infiltration within the lesions in the aortic sinus compared with vehicle treatment (10.5 ± 2.3 cells vs. 13.2 ± 3.7 cells, n = 18 each, $p < 0.05$) (Fig. 2C).

We confirmed that aortic expression of DPP-4 and GLP-1 receptor messenger ribonucleic acid (mRNA) was not significantly different between the DFS and vehicle groups (DPP-4 p = 0.83; GLP-1 receptor p = 0.95) (Fig. 2D). DFS treatment significantly reduced the aortic mRNA expression levels of interleukin (IL)-6, IL-1-beta, tumor necrosis factor-alpha, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (Fig. 2D), but not that of IL-10 (p = 0.72) (Fig. 2D).

DFS IMPROVED ENDOTHELIAL FUNCTION. ApoE-deficient mice were treated with DFS, vehicle, or standard diet for 7 weeks (Fig. 3A, Online Table 2). DFS treatment significantly increased fasting plasma levels of active GLP-1 compared with the vehicle and control groups (DFS 16.7 ± 3.6 pg/ml, vehicle 9.9 ± 2.8 pg/ml, control 10.6 ± 2.7 pg/ml, $p < 0.001$) (Fig. 3B). Ingestion of the HFD worsened the vascular endothelium–dependent relaxation in response to acetylcholine. However, DFS significantly improved the vascular endothelium–dependent relaxation in response to acetylcholine compared with the vehicle group (89.9 ± 3.9% vs. 79.2 ± 4.3% relaxation at 10^{-4} mol/l acetylcholine, n = 7 each, $p < 0.05$) (Fig. 3C). In contrast, the vascular endothelium–independent relaxation in response to sodium nitroprusside did not differ among the 3 groups (data not shown). We found that DFS treatment significantly increased endothelial nitric oxide synthase (eNOS) phosphorylation compared with the vehicle (1.46 ± 0.16-fold, n = 7 each, $p < 0.05$) (Fig. 3D), although the total eNOS protein was not different between the 2 groups.

Cell culture studies. **ENHANCED GLP-1 ACTIVITY BY DFS INCREASED CYCLIC ADENOSINE MONOPHOSPHATE (CAMP) LEVELS, LEADING TO REDUCED EXPRESSION OF LIPOPOLYSACCHARIDE (LPS)–INDUCED INFLAMMATORY CYTOKINES AND THE PRODUCTION OF REACTIVE OXYGEN SPECIES IN CULTURED HUMAN MACROPHAGES.** Reverse transcriptase polymerase chain reaction and Western blot analyses showed the expression of DPP-4 and GLP-1 receptor on human monocytes, THP-1 cell–derived macrophages (days 1 and 4), HCAECs, and human aortic endothelial cells (Figs. 4A and 4B).

As shown in Figure 4C, LPS-induced inflammation (evaluated by IL-6 production) was significantly attenuated by 20 to 100 pmol/l active GLP-1, but not by the physiological levels of active GLP-1 (10 pmol/l) alone. Co-incubation of macrophages with DFS and physiological levels of GLP-1 significantly reduced LPS-induced production of inflammatory cytokines and monocyte chemoattractant protein-1 in a dose-dependent manner of DFS (Figs. 4D to 4G). In contrast, DFS and GLP-1 had no effect on LPS-induced production of IL-10 (p = 0.88) (Fig. 4H).

The IL-4-induced macrophage production of IL-10 was significantly increased compared with control. However, DFS and GLP-1 had no effect on the IL-4–induced production of IL-10 compared with DFS or GLP-1 alone (Online Fig. 1A). The mRNA levels of CD204 and transforming growth factor–beta were also significantly induced by IL-4 in macrophages, but they were not significantly different between DFS and GLP-1 and DFS or GLP-1 alone (Online Fig. 1B).

Pre-treatment with DFS significantly enhanced the cytosolic levels of cAMP induced by GLP-1 (after 60 min, +20.1 ± 6.8%, $p < 0.05$; area under the curve +24.0 ± 1.7%, $p < 0.001$) (Fig. 5A), resulting in a significant inhibition of LPS-induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and c-jun N-terminal kinase (JNK) compared with GLP-1 alone (ERK 1/2: after 5 min, −49.4 ± 16.5%, $p < 0.01$, after 15 min, −42.6 ± 11.5%, $p < 0.01$; JNK: after 5 min, −65.8 ± 6.7%, $p < 0.001$, after 15 min, −60.4 ± 9.3%, $p < 0.01$) (Figs. 5B and 5C). DFS and GLP-1 treatment significantly inhibited LPS-induced phosphorylation of inhibitor of kappa B kinase–beta and nuclear factor–kappa B (NF-κB) p65 nuclear translocation compared with GLP-1 alone (inhibitor of kappa B kinase–beta −56.7 ± 3.5%, $p < 0.001$; NF-κB −70.8 ± 4.8%, $p < 0.001$) (Figs. 5F and 5H). The addition of MDL-12,330A, a specific adenylyl cyclase inhibitor, and H89, a specific protein kinase A (PKA) inhibitor, significantly attenuated the inhibitory effects of DFS and GLP-1 on LPS-induced phosphorylation of ERK 1/2, JNK, and inhibitor of kappa B kinase–beta, and NF-κB activation (Figs. 5D, 5E, 5G, and 5H). Furthermore, the production of

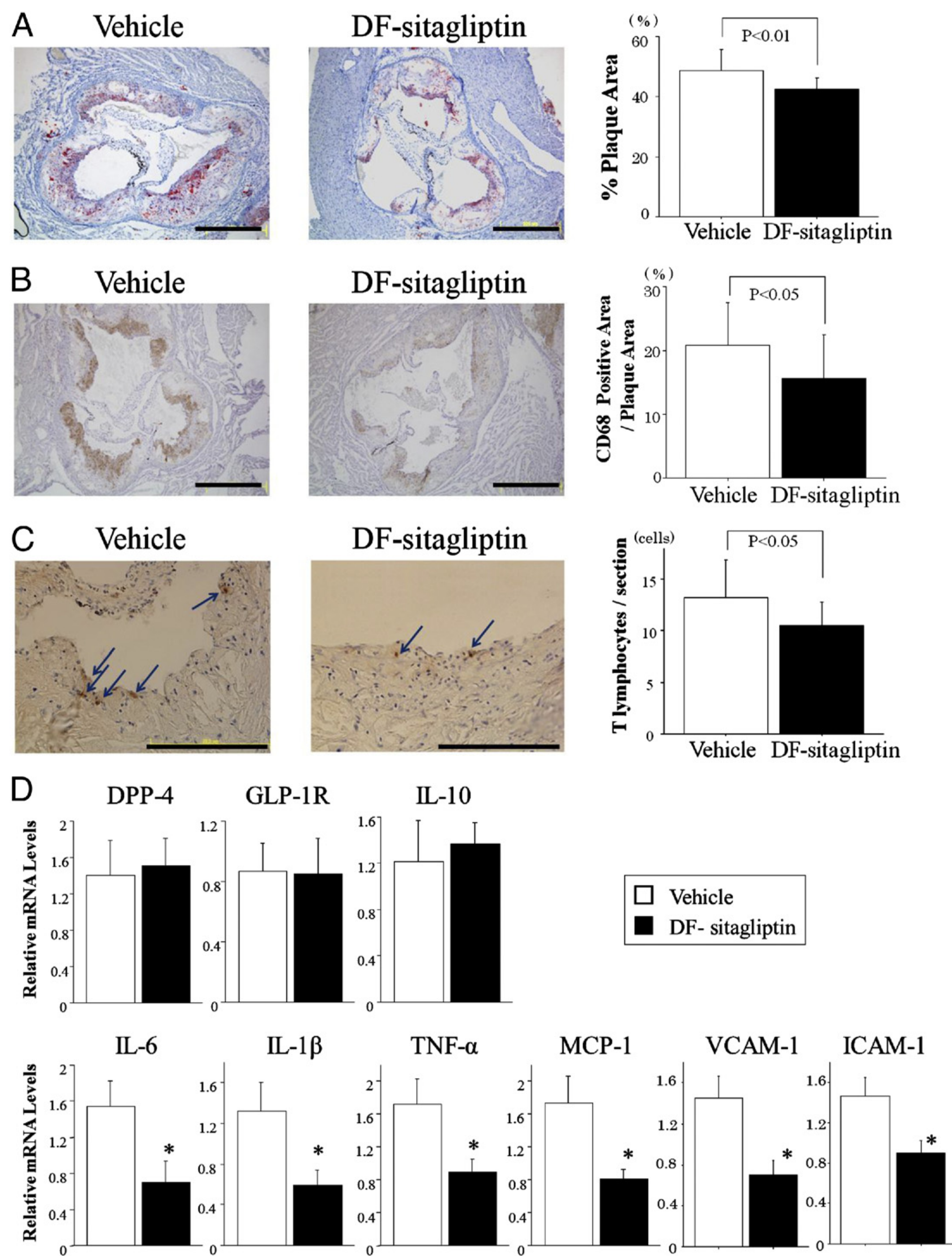

Figure 2 **DFS Reduces Atherosclerotic Lesion Formation, Amount of Macrophages Within Plaque, and Proinflammatory Cytokines in ApoE-Deficient Mice**

(A to C) Histological analyses of **(A)** atherosclerotic lesion area (Oil Red O stain), **(B)** amount of macrophages in plaque (anti-CD68 stain), and **(C)** T lymphocyte infiltration within plaque lesions (anti-CD90 stain) in aortic sinuses of vehicle-treated and des-fluoro-sitagliptin (DFS)–treated apolipoprotein E (apoE)–deficient mice (n = 18 each, p values by Student's *t* tests). Scale bars = 500 μm in **A** and **B** and 200 μm in **C**. **Arrows** in **C** point to infiltrating T lymphocytes. **(D)** Relative messenger ribonucleic acid (mRNA) expression levels of dipeptidyl peptidase (DPP)-4, glucagon-like peptide (GLP)-1 receptor, interleukin (IL)-10, IL-6, IL-1-beta, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein (MCP)-1, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 measured by quantitative real-time reverse transcriptase polymerase chain reaction in the aortas (n = 6 each) and expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Values represent fold of the mean value measured in controls. $*p < 0.05$ versus vehicle. Values are mean ± SD.

reactive oxygen species from macrophages treated with DFS and GLP-1 as significantly decreased compared with those treated with DFS or GLP-1 alone (Online Fig. 2).

DFS-INDUCED ENHANCEMENT OF GLP-1 ACTIVITY INCREASED ENOS PHOSPHORYLATION AND INHIBITED PREMATURE SENESCENCE AND OXIDATIVE STRESS–INDUCED APOPTOSIS OF HCAECS. Both GLP-1 alone and GLP-1 combined with DFS (at 2 and 5 min after the addition of GLP-1) significantly increased eNOS phosphorylation in HCAECs. However, GLP-1 combined with DFS succeeded in significant continuation of eNOS phosphorylation at 10 min (1.47 ± 0.04-fold, $p < 0.001$) (Fig. 6A). Furthermore, the addition of MDL-12,330A and H89 significantly suppressed the enhancing effects of DFS on GLP-1–induced eNOS phosphorylation (Fig. 6B).

Cotreatment with DFS and GLP-1 significantly decreased the number of senescence-associated beta-galactosidase-positive cells compared with GLP-1 alone

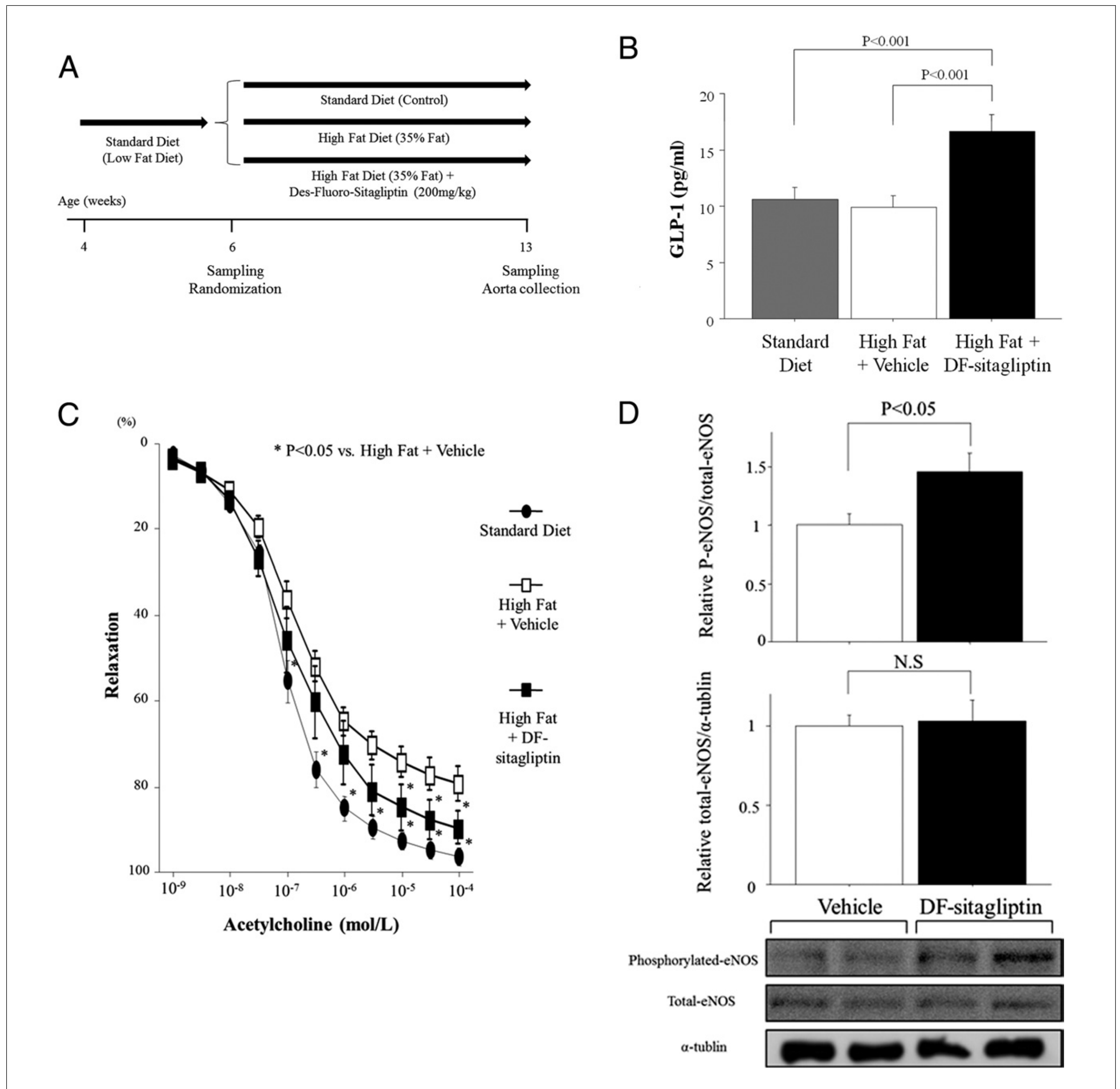

Figure 3 **DFS Improves Endothelial Function in ApoE-Deficient Mice**

(A) Schematic diagram of the study protocol. **(B)** Fasting plasma levels of active GLP-1 in the high-fat diet (HFD) with DFS group (n = 7), HFD with vehicle group (n = 7), and standard diet group (n = 8). **(C)** Vascular endothelium–dependent relaxation to acetylcholine (ACh). **(D)** Western blot analysis using protein samples from the aortas of mice in the vehicle and DFS groups (n = 7 each). Values are mean ± SD. Abbreviations as in Figure 1.

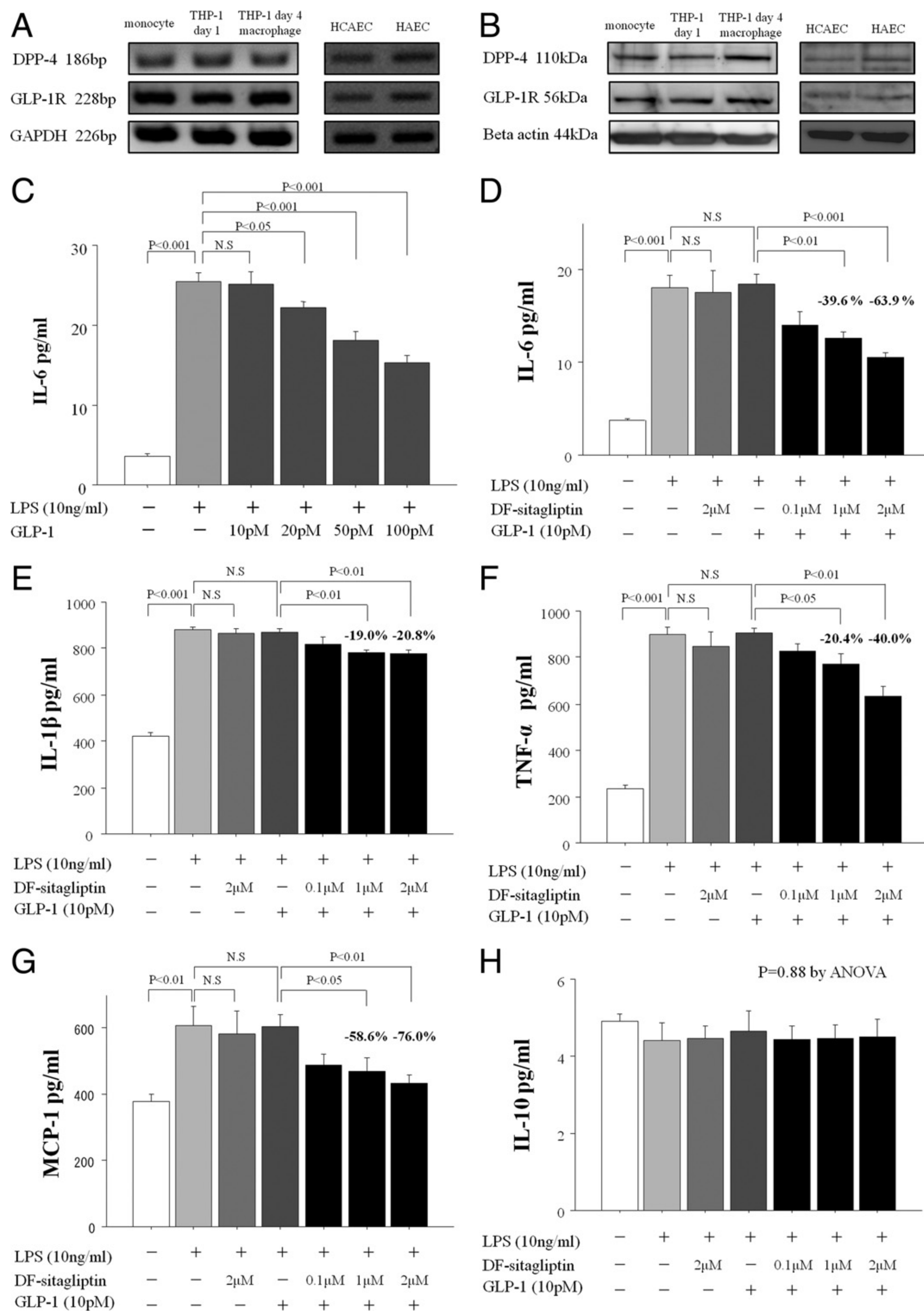

Figure 4 **Anti-Inflammatory Effects of DFS in Cultured Human Macrophages**

Reverse transcriptase polymerase chain reaction (RT-PCR) **(A)** and Western blot **(B)** analyses indicated that human monocytes, THP-1 cell–derived macrophages, human coronary artery endothelial cells, and human aortic endothelial cells expressed dipeptidyl peptidase–4 (DPP-4) and GLP-1 receptor. **(C)** Effect of treatment of THP-1–derived macrophages with the indicated concentrations of GLP-1 (10 to 100 pmol/l), followed by stimulation with 10 ng/ml lipopolysaccharide (LPS) on the production of IL-6 (n = 5 or 6). **(D to H)** Effects of co-incubation of macrophages with GLP-1 (10 pmol/l) and DFS on LPS-induced production of **(D)** IL-6, **(E)** IL-1-beta, **(F)** TNF-α, **(G)** MCP-1, and **(H)** IL-10. Values are means of percent reduction compared with GLP-1 alone. Bars are mean ± SEM values on 5 or 6 experiments. Abbreviations as in Figures 1 and 2.

(73.4 ± 2.4% vs. 88.6 ± 2.8%, $p < 0.001$) (Fig. 6C). Cotreatment with GLP-1 and DFS significantly decreased the percent of apoptotic HCAECs induced by 100 μmol/l H_2O_2 (annexin-V^+/propidium iodide$^-$) compared with GLP-1 alone (11.0 ± 0.6% vs. 23.6 ± 1.4%, $p < 0.001$) (Fig. 7A). Pre-treatment with L-NAME, the eNOS inhibitor, significantly attenuated the inhibitory effects of DFS and GLP-1 on endothelial senescence and apoptosis (Figs. 6D and 7B). The intracellular defensive factors against oxidative stress (Cu-Zn-

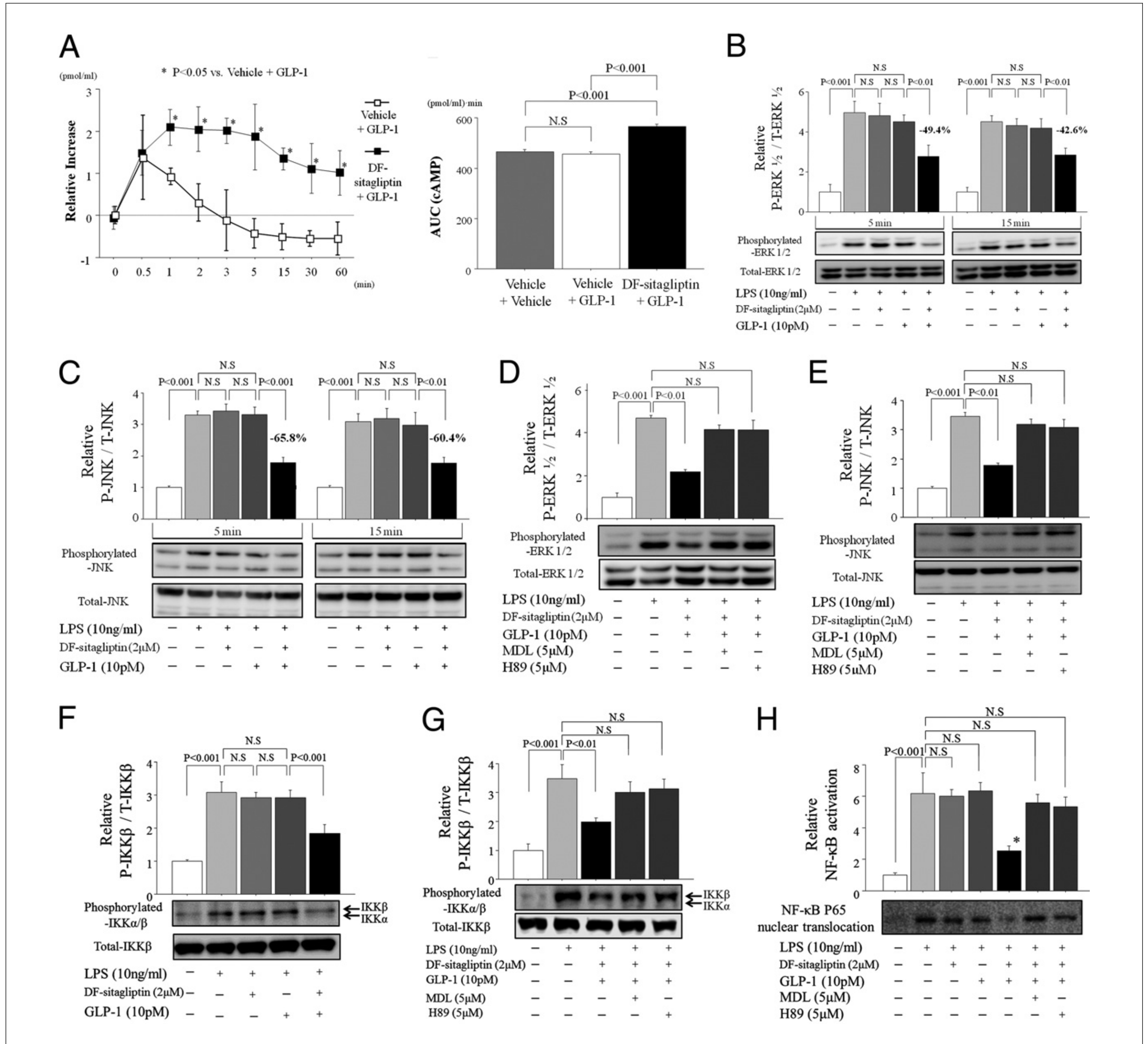

Figure 5 **Effect of DFS on Cytosolic cAMP Levels and ERK 1/2 and JNK Phosphorylation and NF-κB Activation in Cultured Human Macrophages**

(A) Effects of pre-treatment of THP-1–derived macrophages with 2 μmol/l DFS followed by 10 pmol/l GLP-1 (n = 6 each) on cytosolic levels of cyclic adenosine monophosphate (cAMP). AUC = area under the curve. **(B,C)** Effects of pre-treatment of macrophages with 2 μmol/l DFS followed by 10 pmol/l GLP-1 and 10 ng/ml lipopolysaccharide (LPS) on intracellular levels of extracellular signal-regulated kinase (ERK) 1/2 **(B)** and c-jun N-terminal kinase (JNK) **(C)** at 5 and 15 min and inhibitor of kappa B kinase–beta (IKKβ) **(F)** at 15 min after LPS stimulation. **(D,E,G)** Effects of treatment of macrophages with 5 μmol/l MDL-12,330A, 5 μmol/l H89, DFS (2 μmol/l) and GLP-1 (10 pmol/l) on ERK 1/2 **(D)** and JNK **(E)** activation at 5 min and IKKβ **(G)** at 15 min after LPS stimulation (n = 6 each). **(B,D) (Top)** Immunoblot with antiphosphorylated ERK 1/2 (upper band 44 kDa, lower band 42 kDa); **(bottom)** immunoblot with anti–ERK 1/2 (upper band 44 kDa, lower band 42 kDa) as control (representative samples). **(C,E) (Top)** Immunoblot with antiphosphorylated JNK (upper band 54 kDa, lower band 46 kDa); **(bottom)** immunoblot with anti-JNK (upper band 54 kDa, lower band 46 kDa) as control (representative samples). Values are means of the percent reduction compared with GLP-1 alone. **(F,G) (Top)** Immunoblot with antiphosphorylated IKKα/β (upper band [IKKβ] 87 kDa, lower band [IKKα] 85 kDa); **(bottom)** anti-IKKβ (band 87 kDa) as control (representative samples). **(H)** Effects of treatment of nuclear extracts from macrophages with 5 μmol/l MDL-12,330A, 5 μmol/l H89, DFS (2 μmol/l), and GLP-1 (10 pmol/l) followed by 10 ng/ml LPS on activation nuclear factor–kappa B (NF-κB) at 20 min after LPS stimulation (n = 6 each). Immunoblot with anti-NF-κB p65 nuclear translocation (band 65 kDa). Bars represent the mean ± SEM values. Abbreviations as in Figure 1.

superoxide dismutase, glutathione peroxidase, and thioredoxin) were significantly increased in HCAECs treated with DFS and GLP-1 compared with those treated with DFS or GLP-1 alone (Fig. 7C).

Furthermore, GLP-1 combined with DFS significantly reduced the mRNA levels of basal expression and those induced by IL-1-beta of intercellular adhesion molecule–1 (basal $-11.0 \pm 4.5\%$, $p < 0.05$; IL-1-beta $-20.0 \pm 7.5\%$; $p < 0.05$) and vascular

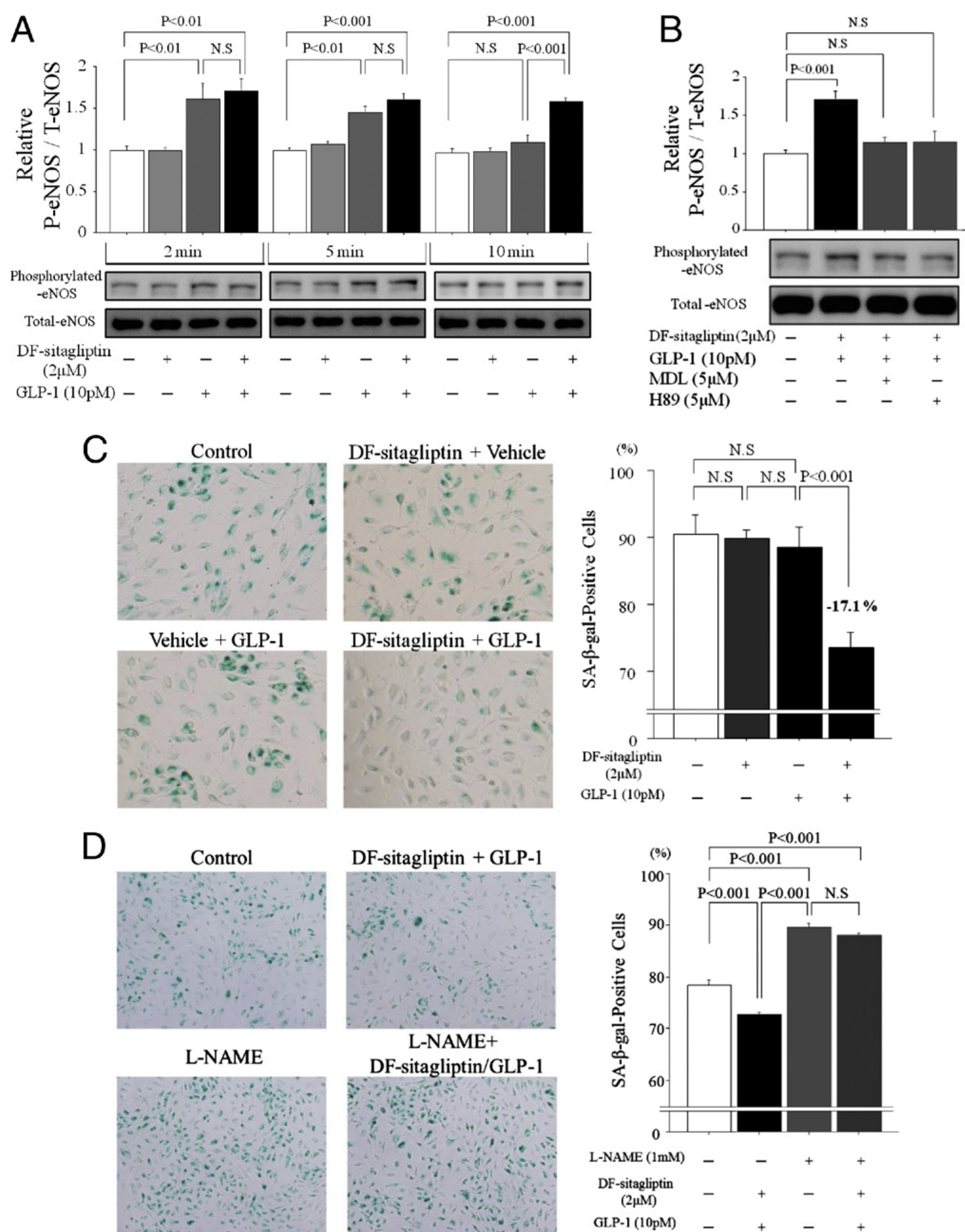

Figure 6 **Effects of DFS on Endothelial Cell Function**

(A) Human coronary artery endothelial cells (HCAECs) were pre-treated with DFS (2 μmol/l) or vehicle for 1 h, then treated with GLP-1 (10 pmol/l) or vehicle. The phosphorylation of endothelial nitric oxide synthase (eNOS) at 2, 5, and 10 min after GLP-1 administration was quantified by Western blots (n = 6 each). **(B)** HCAECs were pre-treated with 5 μmol/l MDL-12,330A, 5 μmol/l H89, or vehicle for 30 min before the addition of GLP-1. The phosphorylation of eNOS at 2 min after GLP-1 administration was quantified by Western blots (n = 6 each). **(Top)** Immunoblot with anti-phosphorylated eNOS (band 140 kDa); **(bottom)** immunoblot with anti-eNOS (band 140 kDa) as control (representative samples). **(C)** HCAECs were treated with or without DFS (2 μmol/l) and GLP-1 (10 pmol/l). **(D)** HCAECs were pre-treated with or without 1 mmol/l L-NAME for 30 min before treatment with GLP-1.The medium was changed, and DFS, GLP-1, and L-NAME were added every day at the indicated concentrations. At the 10th **(C)** or 5th **(D)** day, HCAECs were fixed, and the proportion of senescence-associated beta-galactosidase staining was determined (n = 6 each). Values are means of the percent reduction compared with GLP-1 alone. **(D)** Values are means of the percent reduction compared with GLP-1 alone. Values are mean ± SEM. Abbreviations as in Figure 1.

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cell adhesion molecule–1 (basal −13.1 ± 5.7%, $p < 0.05$; IL-1-beta −28.0 ± 10.3%, $p < 0.05$) compared with GLP-1 alone (Online Figs. 3A and 3B).

DFS MODULATED THE PROINFLAMMATORY RESPONSE IN PERITONEAL MACROPHAGES. In apoE-deficient mice, the HFD significantly induced the mRNA expression of

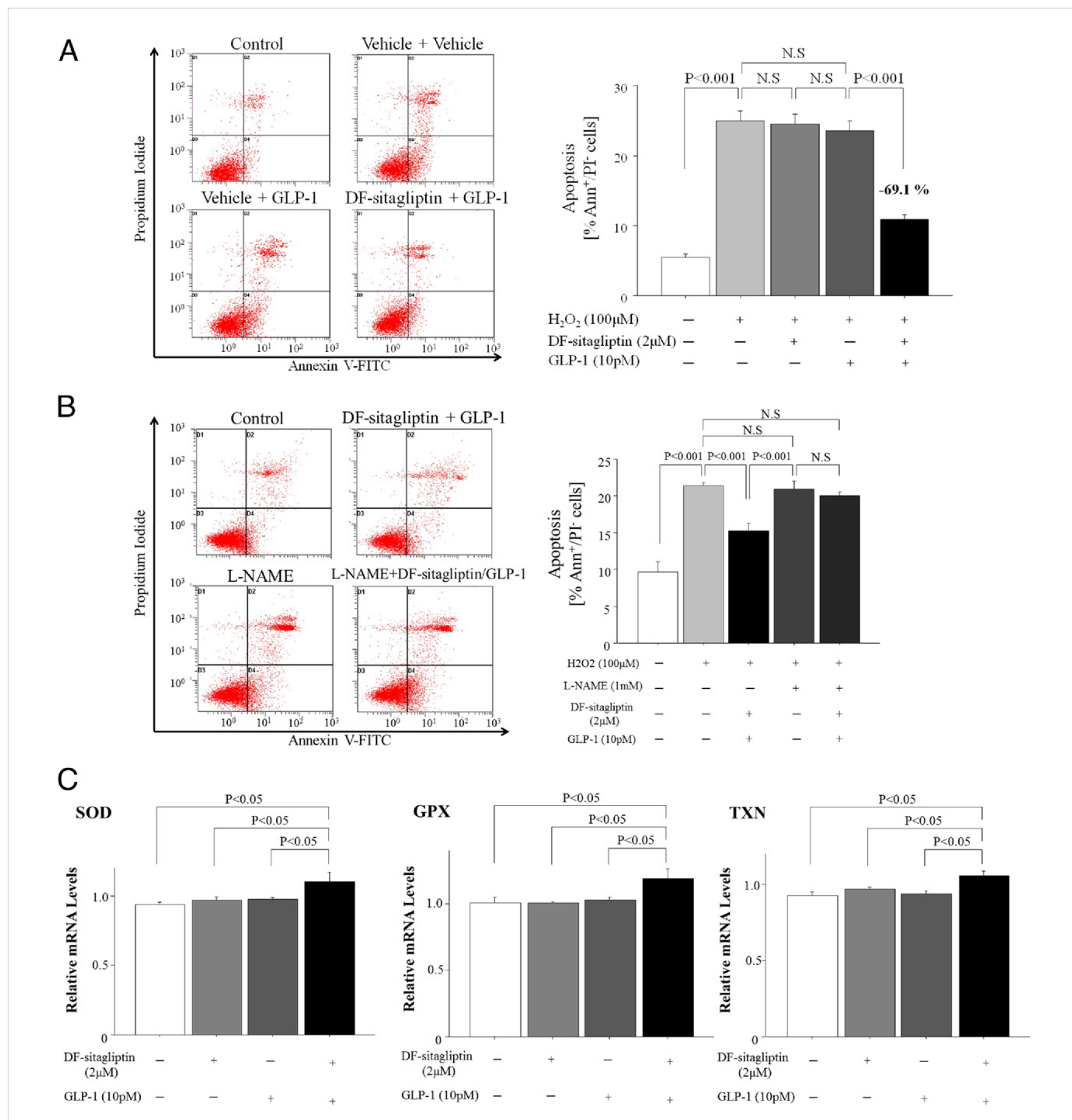

Figure 7 **Effects of DFS on Apoptosis**

(A) Human coronary artery endothelial cells (HCAECs) pre-treated with DFS (2 μmol/l) or vehicle for 1 h, then treated with GLP-1 (10 pmol/l) or vehicle for 1 h, followed by stimulation with 100 μmol/l H_2O_2 for 30 min (n = 6 each). **(B)** HCAECs were pre-treated with or without 1 mmol/l L-NAME for 30 min before treatment with GLP-1. Fluorescence-activated cell sorting derived dot plot of HCAECs stained with annexin-V-FITC and propidium iodide (PI). Apoptotic cells were identified by annexin-V-FITC$^+$/PI$^-$ staining (lower right quadrants). Values are means of the percent reduction compared with GLP-1 alone. **(C)** The defensive factors against oxidative stress were significantly increased in HCAECs treated with DFS (2 μmol/l) and GLP-1 (10 pmol/l). Relative messenger ribonucleic acid (mRNA) expression levels of Cu-Zn-superoxide dismutase (SOD), glutathione peroxidase (GPX), and thioredoxin (TXN) measured by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) in HCAECs treated with or without DFS (2 μmol/l) for 1 h, followed by treatment with or without GLP-1 (10 pmol/l) for 24 h and expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (n = 6 each). Values are mean ± SEM. Abbreviations as in Figure 1.

proinflammatory factors in peritoneal macrophages after 4 weeks ($p < 0.05$). DFS treatment significantly reduced the mRNA expression levels of IL-6, IL-1-beta, and monocyte chemoattractant protein-1 ($p < 0.05$ for each) and tended to reduce the mRNA expression levels of tumor necrosis factor-alpha ($p = 0.31$). DFS did not exhibit significant effects on the mRNA expression levels of IL-10, IL-4, and transforming growth factor–beta (Online Fig. 4).

Human studies. LOW FASTING PLASMA LEVELS OF ACTIVE GLP-1 IN PATIENTS WITH CAD. Online Appendix Table 3 lists the clinical characteristics of patients with or without CAD. The proportion of patients with CAD with fasting levels of active GLP-1 below the detection limit (2.0 pmol/l; $n = 19$) was significantly higher than those without CAD ($n = 8$) ($p < 0.05$). Furthermore, the fasting plasma levels of active GLP-1 in patients with CAD ($n = 81$) were significantly lower than in those without CAD ($n = 92$), excluding patients with undetectable levels of active GLP-1 (3.10 pmol/l [2.40 to 3.62 pmol/l] vs. 4.00 pmol/l [3.10 to 5.90 pmol/l], $p < 0.001$) (Online Fig. 5A). Among patients without DM, the fasting plasma levels of active GLP-1 in patients with CAD ($n = 58$) were also significantly lower than in those without CAD ($n = 70$), excluding patients with undetectable levels of active GLP-1 (3.20 pmol/l [2.60 to 3.70 pmol/l] vs. 3.90 pmol/l [3.00 to 5.20 pmol/l], $p < 0.001$) (Online Fig. 5B). The multivariate logistic regression analysis revealed that the lower active GLP-1 independently correlated with the presence of CAD after adjustment for significant factors by univariate logistic regression (lower group; odds ratio: 5.87; 95% confidence interval: 2.42 to 14.19; $p < 0.001$; Hosmer-Lemeshow test $p = 0.59$) and in the forced-entry models (lower group; odds ratio: 5.62; 95% confidence interval: 2.83 to 11.14; $p < 0.001$; Hosmer-Lemeshow test $p = 0.77$) (Online Table 4).

Discussion

The present study demonstrated that the DPP-4 inhibitor DFS reduced atherosclerosis lesion formation and improved endothelial dysfunction in apoE-deficient mice independent of fasting glucose and lipid profile. Furthermore, when the physiological concentrations of GLP-1 and DFS were used in the in vitro experiments, DFS significantly increased the cytosolic levels of cAMP induced by GLP-1 and consequently reduced the expression of proinflammatory mediators in human cultured macrophages, as well as increasing phosphorylation of eNOS and reducing senescence and apoptosis in cultured HCAECs, compared with GLP-1 alone.

Inflammatory responses mediated by inflammatory cytokines are considered important in all stages of atherosclerosis (7). The present study showed that DFS reduced the mRNA expression levels of proinflammatory mediators in the aortas of apoE-deficient mice. Indeed, the present animal studies showed that DFS decreased the percent area of macrophages and T lymphocytes in the plaques and reduced expression of proinflammatory factors in peritoneal macrophages. Furthermore, coapplication of DFS and GLP-1, but not DFS or GLP-1 alone, significantly reduced the production of reactive oxygen species and proinflammatory mediators in human cultured macrophages and attenuated the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in HCAECs. According to the results shown in Figures 4E and 6D and Online Figures 1 and 3D, treatment with DFS and GLP-1 could exhibit antiatherogenic and anti-inflammatory effects through a possible direct link between the effects of DFS and GLP-1 on macrophages and those on endothelial cells.

Experimental evidence suggests that the control of dysregulated mitogen-activated protein kinase cascade and NF-κB activation could be a potential target in the treatment of atherosclerosis (9,10). Arakawa et al. (4) showed that the GLP-1 receptor agonist exendin-4 reduced atherosclerotic lesion in mice and that this effect may contribute to the inhibition of NF-κB p65 nuclear translocation in macrophages by increased cAMP levels elicited by GLP-1 receptor activation. The present study further showed that DFS significantly increased the level of GLP-1-induced cytosolic cAMP and consequently inhibited the activation of intracellular inflammatory signaling through the cAMP/PKA pathway in human cultured macrophages. These findings suggest that inhibition of the inflammatory activity of macrophages by enhancing GLP-1 receptor activation using DFS could attenuate inflammation and modulate the atherogenic process via the cAMP/PKA pathway.

Nitric oxide, produced by eNOS, plays important roles in vascular biology, including regulation and coordination of endothelial cell function, proliferation, senescence, and apoptosis (11), and exhibits atheroprotective effects (12). PKA activates eNOS in endothelial cells (13). The present study showed that DFS improved HFD-induced endothelial dysfunction by increasing the phosphorylation of eNOS in apoE-deficient mice. Furthermore, the in vitro experiments showed that DFS increased or augmented GLP-1 activity and thereby enhanced the phosphorylation of eNOS through the cAMP/PKA pathway and decreased the endothelial inflammation, senescence, and apoptosis of HCAECs. Pre-treatment with L-NAME significantly attenuated the inhibitory effects of DFS and GLP-1 on endothelial inflammation, senescence, and apoptosis. These results suggest that DPP-4 inhibitors might, at least in part, inhibit atherosclerosis by protecting endothelial functions and preserving endothelial integrity. However, it is not clear whether eNOS phosphorylation could be activated by GLP-1 through the other signaling pathways. Further experiments would be needed to determine the details of intracellular signaling mechanisms of the enhanced eNOS phosphorylation by DFS and GLP-1.

Intraperitoneal glucose tolerance testing showed no differences in plasma glucose and insulin concentrations among all groups in the present study. The results indicate that this mouse model was not diabetic. On oral glucose tolerance testing, differences among plasma glucose levels after glucose load were small but significantly decreased in the DFS group compared with the vehicle group, confirming the enhancement of incretin effects by DFS. The present study also showed that

low levels of fasting active GLP-1 were significantly correlated with the high percent atherosclerotic area in mice model. Furthermore, the human studies demonstrated that the fasting plasma levels of active GLP-1 were significantly lower in patients with CAD than those without CAD (Online Figs. 5A and 5B). According to these results, the endogenous GLP-1 activity augmented by DPP-4 inhibitors could exhibit anti-atherogenic effects.

CD26 is a cell surface protein with DPP-4 enzymatic activity, as well as a multifunctional protein involved in T lymphocytes activation and correlated with the production of T helper 1–type responses (14). There is a concept emerging from numerous lines of evidence that macrophages can develop into different subsets: classic macrophages, activated by T helper 1–type responses, or alternative macrophages, activated by T helper 2–type responses (15). In a previous study, DPP-4 inhibition in vivo by genetic and pharmacological blockade did not affect T lymphocyte–dependent immune responses (16). Treatment with sitagliptin, a DPP-4 inhibitor, has been reported to have no clinical effect on T lymphocyte activation in patients with type 2 DM (17). Therefore, the standard therapy of sitagliptin might not affect T lymphocyte functions. Furthermore, the present study demonstrated that DFS and GLP-1 acted on the intracellular signaling cascade, although it was not clear that DFS and GLP-1 affected macrophage phenotypic polarity shift from classic to alternative macrophages. However, because DPP-4 inhibitors are relatively new drugs, further in vivo and in vitro studies are needed for complete evaluations of the long-term effects of DPP-4 inhibitors on T lymphocyte and macrophage functions and vascular wall cells.

Study limitations. The plasma levels of active GLP-1 were measured in only a small number of patients. Medications commonly used for the treatment of patients with chronic diseases may affect plasma levels of active GLP-1. Thus, a multicenter trial that includes a large population is warranted to further examine the association between plasma levels of active GLP-1 and the presence of CAD and the antiatherogenic effects of DPP-4 inhibitors in humans.

Conclusions

The present study demonstrated that DFS, a DPP-4 inhibitor, significantly improved endothelial function and reduced atherosclerotic lesion formation in apoE-deficient mice on an HFD, independent of lipid profile. The antiatherogenic effects of DPP-4 inhibitors on macrophages and endothelial cells through the enhancement of GLP-1 activity could be beneficial to cardiovascular function.

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Key Words: atherosclerosis ■ dipeptidyl peptidase-4 inhibitors ■ endothelium ■ glucagon-like peptide 1 receptor ■ inflammation ■ receptors.

APPENDIX

For an expanded Methods section and supplemental figures and tables, please see the online version of this article.