Dipeptidyl Peptidase 4 Inhibitor Sitagliptin Protects Endothelial Function in Hypertension Through a Glucagon–Like Peptide 1–Dependent Mechanism

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Abstract—Sitagliptin, a selective dipeptidyl peptidase 4 inhibitor, inhibits the inactivation and degradation of glucagon like peptide 1 (GLP-1), which is used for the treatment of type 2 diabetes mellitus. However, little is known about the role of GLP-1 in hypertension. This study investigated whether the activation of GLP-1 signaling protects endothelial function in hypertension. Two-week sitagliptin treatment (10 mg/kg per day, oral gavage) improved endothelium-dependent relaxation in renal arteries, restored renal blood flow, and reduced systolic blood pressure in spontaneously hypertensive rats. In vivo sitagliptin treatment elevated GLP-1 and GLP-1 receptor expressions, increased cAMP level, and subsequently activated protein kinase A, liver kinase B1, AMP-activated protein kinase-α and endothelial NO synthase in spontaneously hypertensive rat renal arteries. Inhibition of GLP-1 receptor, adenylyl cyclase, protein kinase A, AMP-activated protein kinase-α, or NO synthase reversed the protective effects of sitagliptin. We also demonstrate that GLP-1 receptor agonist exendin 4 in vitro treatment had similar vasoprotective effects in spontaneously hypertensive rat renal arteries and increased NO production in spontaneously hypertensive rat aortic endothelial cells. Studies using transient expressions of wild-type and dominant-negative AMP-activated protein kinase-α2 support the critical role of AMP-activated protein kinase-α in mediating the effect of GLP-1 in endothelial cells. Ex vivo exendin 4 treatment also improved endothelial function of renal arteries from hypertensive patients. Our results elucidate that upregulation of GLP-1 and related agents improve endothelial function in hypertension by restoring NO bioavailability, suggesting that GLP-1 signaling could be a therapeutic target in hypertension-related vascular events. (Hypertension. 2012;60:833-841.) ● Online Data Supplement

Key Words: dipeptidyl peptidase 4 ▪ endothelium-dependent relaxation ▪ glucagon-like peptide 1 ▪ NO ▪ protein kinases ▪ spontaneously hypertensive rats

Hypertension is caused by pathological changes in renal and vascular structure and function involved in blood pressure regulation.1 Hypertension can cause renal damage if it is not properly controlled.2 The impaired vasodilator response is a risk factor for renal function loss in patients with essential hypertension.3 Persistent hypertension alters functional characteristics of vascular endothelial cells and is associated with impaired vasodilatory function.4 Diminished production and function of endothelium-derived NO leads to endothelial dysfunction,5 a crucial initial step culminating in vascular events in hypertension.

Dipeptidyl peptidase 4 (DPP-4), also known as CD26, is a ubiquitous enzyme detectable in the endothelium.6 Glucagon-like peptide 1 (GLP-1) produced by L-type cells in the intestine, is a substrate for DPP-4.7 GLP-1 improves glucose use in patients with type 2 diabetes mellitus by increasing insulin secretion and inhibiting glucagon secretion.8,9 Sitagliptin, a highly selective DPP-4 inhibitor,10 inhibits the inactivation and degradation of GLP-1,11 which is used for the treatment of type 2 diabetes mellitus as monotherapy or in combination with other antiglycemic agents, such as metformin.12

The effect of GLP-1 on blood pressure has been reported in both animal and human hypertension.13,14 Treatment with GLP-1 receptor (GLP-1R) agonists leads to a transient blood pressure increase attributed to the impact on sympathetic
outflow. However, continuous infusion of GLP-1 produces a small but insignificant reduction of blood pressure in patients with type 2 diabetes mellitus. Moreover, chronic administration of recombinant GLP-1 prevents the development of hypertension and improves endothelial function in Dahl salt-sensitive rats. By contrast, the effect of DPP-4 inhibition on blood pressure is largely unknown, although limited studies show that DPP-4 inhibition by sitagliptin produces a small blood pressure–lowering effect in nondiabetic patients with hypertension on stable antihypertensive therapy. The present study investigated whether DPP-4 inhibition could ameliorate endothelial dysfunction in renal arteries from hypertensive animals and patients, as well as possible signaling mechanisms involved.

**Materials and Methods**

A supplemental Methods section can be found in the online-only Data Supplement.

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**Figure 1.** Sitagliptin lowers blood pressure and increases renal blood flow in spontaneously hypertensive rats (SHRs). **A,** Systolic blood pressure (SBP) in vehicle and sitagliptin-treated Wistar-Kyoto rats (WKYs) and SHRs. **B,** Renal blood flow in WKY vehicle (1) rats or in vehicle (3) and sitagliptin-treated SHRs (2). Data are mean ± SEM. *P < 0.05 vs WKY vehicle, #P < 0.05 vs SHR vehicle. n = 4.
**Measurement and Analysis of Renal Blood Flow by MRI Acquisition Procedure**

MRI studies were performed using a 3T clinical whole-body imaging system (Achieva, Philips Healthcare, Best, the Netherlands).19

**Renal Artery Preparation and Functional Studies**

Rats were euthanized by CO₂ suffocation, and renal interlobar arteries were removed and placed in ice-cold Krebs solution. Arteries were prepared, and changes of isometric tension were recorded.20,21

**Western Blot Analysis**

Protein expression levels of GLP-1, GLP-1R, phospho-PKA C (protein kinase A catalytic subunit, Thr197), phospho-LKB1 (liver kinase B1) at Thr172, phospho-AMP-activated protein kinase (AMPKα at Thr172), GAPDH, and eNOS were detected by Western blotting.

**NO Measurement**

Intracellular NO production was monitored using a fluorescent NO indicator 4-aminomethylamino-2,7-difluorofluorescein diacetate22 and the Total Nitric Oxide Assay kit.

**Data Analysis**

Results represent mean±SEM from different rats or patients. Statistical significance was determined by 2-tailed Student t test or 1-way ANOVA followed by the Bonferroni post hoc test when >2 treatments were compared. P values <0.05 indicate statistically significant difference.

**Results**

**Sitagliptin Treatment Lowers Blood Pressure and Increases Renal Blood Flow in Spontaneously Hypertensive Rats**

Ambulatory arterial pressure in conscious, unrestrained spontaneously hypertensive rats (SHRs) was monitored by radiotelemetry. The ambulatory systolic blood pressures (SBP) were significantly lower in 2-week sitagliptin-treated SHRs compared with vehicle-treated SHRs (averaged SBP, 160±8 versus 180±5 mm Hg; n=4 each group), whereas sitagliptin treatment did not alter SBPs in Wistar-Kyoto rats (WKYS; averaged SBP, 120±4 versus 119±4 mm Hg; n=4 each group; Figure 1A). This is further confirmed by the direct...
Sitagliptin Improves Endothelial Function in SHR Renal Arteries

Two-week sitagliptin administration increased the plasma concentration of GLP-1 in WKYs and SHRs (Figure S2A), as well as GLP-1 and GLP-1R (Figure S2B and S2C) expressions in renal arteries. Treatment with sitagliptin markedly increased acetycholine-induced endothelium-dependent relaxation (EDR) in SHR renal arteries without affecting those in WKYs (Figure 2A; pD2, 6.66±0.12 in SHRs versus 7.22±0.10 in WKYs and EMax%: 25.8±1.7 in SHRs versus 76.3±5.7 in WKYs; P<0.05). By contrast, endothelium-independent relaxations to sodium nitroprusside were similar among all of the groups (Figure 2B). The phosphorylations of PKA C (Figure 2C), LKB1 (Figure 2D), AMPKα (Figure 2E), and eNOS (Figure 2F) were elevated in renal arteries from WKYs and SHRs after sitagliptin treatment, which were reversed by SQ22536 (100 μmol/L) and H89 (1 μmol/L) on endothelium-dependent relaxation (EDR) in renal arteries from sitagliptin-treated SHRs. Data are mean±SEM. *P<0.05 vs vehicle (Veh), #P<0.05 vs sitagliptin. n=4 for Western blotting; n=8 for relaxations.

measurement of SBP with a direct catheter in anesthetized rats (Figure S1A, available in the online-only Data Supplement) and the tail-cuff method (Figure S1B). However, sitagliptin treatment did not significantly lower mean arterial blood pressure or diastolic blood pressure in all groups of rats. It is noted that the effect of sitagliptin on SBP in SHRs was a step decrease that occurred between days 3 and 4 and that there was no diurnal rhythm in control and sitagliptin-treated rats (Figure S1C). Renal blood flow (RBF) reduction in SHRs was restored by 2-week sitagliptin therapy (Figure 1B).

Sitagliptin Improves Endothelial Function in SHR Renal Arteries

Two-week sitagliptin administration increased the plasma concentration of GLP-1 in WKYs and SHRs (Figure S2A), as well as GLP-1 and GLP-1R (Figure S2B and S2C) expressions in renal arteries. Treatment with sitagliptin markedly increased acetycholine-induced endothelium-dependent relaxation (EDR) in SHR renal arteries without affecting those in WKYs (Figure 2A; pD2, 6.66±0.12 in SHRs versus 7.22±0.10 in WKYs and EMax%: 25.8±1.7 in SHRs versus 76.3±5.7 in WKYs; P<0.05). By contrast, endothelium-independent relaxations to sodium nitroprusside were similar among all of the groups (Figure 2B). The phosphorylations of PKA C (Figure 2C), LKB1 (Figure 2D), AMPKα (Figure 2E), and eNOS (Figure 2F) were elevated in renal arteries from WKYs and SHRs after sitagliptin treatment, which were reversed by SQ22536 (100 μmol/L; adenylyl cyclase inhibitor) and H89 (1 μmol/L; PKA inhibitor; Figure 3A through 3D) but not those of PKA C and LKB1 (Figure 3A and 3D) and exendin 9-39 (100 nmol/L; GLP-1R antagonist; Figure 4A through 4D). The increased phosphorylations of AMPKα and eNOS (Figure 4C and 4D) but not those of PKA C and LKB1 (Figure 4A and 4B) were reversed by compound C (10 μmol/L; AMPKα inhibitor). SQ22536 (100 μmol/L) and H89 (1 μmol/L; Figure 3E), exendin 9-39 (100 nmol/L), compound C (10 μmol/L), and Nω-nitro-L-arginine methyl ester (100 μmol/L; NO synthase inhibitor; Figure 4E) also inhibited the improved EDR. Sitagliptin treatment in vivo increased cAMP levels in SHR renal arteries, which were inhibited by exendin 9-39 (100 nmol/L) and SQ22536 (100 μmol/L) but not by compound C (10 μmol/L; Figure S3).
Exendin 4 Improves Endothelium-Dependent Relaxation in SHR Renal Arteries

GLP-1R agonist exendin 4 (10 nmol/L; 12 hours) increased acetylcholine-induced EDR in SHR renal arteries, which were reversed by co-incubation with SQ22536 (100 μmol/L) and H89 (1 μmol/L; Figure 5A) or compound C (10 μmol/L) and Nω-nitro-L-arginine methyl ester (l-NAME; 100 μmol/L; Figure 5B) or exendin 39 (100 nmol/L) and GLP-1R antibody (2.5 μg/mL; Figure 5C). By contrast, exendin 4 treatment for 12 hours had no effect on EDR in WKys (Figure 5D).

Exendin 4 Increases AMPKα and eNOS Phosphorylations and Stimulates NO Production in SHR Aortic Endothelial Cells

Exendin 4 (10 nmol/L) stimulated NO production in primary SHR aortic endothelial cells, which was inhibited by pretreatment with exendin 39-39 (100 nmol/L), SQ22536 (100 μmol/L), H89 (1 μmol/L), compound C (10 μmol/L), or Nω-nitro-L-arginine methyl ester (100 μmol/L; Figure S4 and S5). Twelve-hour ex vivo treatment with either exendin 4 (10 nmol/L) or sitagliptin (10 μmol/L) increased the cGMP level in SHR renal arteries (Figure S6). Transient overexpression of AMPKα2 by wild-type AMPKα2 further increased AMPKα and eNOS phosphorylations (Figure 7) and NO production (Figure S4D) in response to exendin 4 in endothelial cells, whereas suppression of the AMPK activity by dominant-negative AMPKα2 (K45R mutated) inhibited such effects. The level of AMPKα2 increased significantly in SHR endothelial cells by expression of wild-type AMPK but not dominant-negative AMPK (Figure S8).

Sitagliptin Ameliorates Endothelial Dysfunction in Renal Arteries From Hypertensive Patients

EDRs were impaired in renal arteries from hypertensive patients compared with those from normotensive patients, whereas exendin 4 (10 nmol/L; 12 hours) improved EDRs in renal arteries from hypertensive patients (Figure 6A). The reduced GLP-1R level (Figure 6B) and decreased phosphorylations of PKA C, LKB1, AMPKα, and eNOS (Figure 6C through 6F) were elevated by exendin 4 in these arteries.

Discussion

The present study demonstrated a functional importance of GLP-1 and GLP-1R in the regulation of endothelial function in SHR renal vasculature. The major novel findings include: 1) DPP-4 inhibition restores endothelial function by improving AMPKα and eNOS phosphorylations; 2) GLP-1R signaling is essential for the AMPKα-eNOS pathway in endothelial cells; and 3) Sitagliptin treatment improves EDRs by upregulating AMPKα and eNOS expressions.
The present study shows that GLP-1R expression was reduced in SHR renal arteries, whereas in vivo sitagliptin treatment increased the expression of GLP-1R, supporting that DPP-4 inhibition restores the expression and function of GLP-1/GLP-1R in SHR arteries. However, the mechanism of GLP-1R downregulation in hypertension needs to be further elucidated.

Figure 5. Exendin 4 ameliorates endothelial dysfunction in spontaneously hypertensive rat (SHR) renal arteries. Reversal of the improved endothelium-dependent relaxation (EDR) in exendin 4 (10 nmol/L, 12 hours)-treated SHR renal arteries by cotreatment with (A) SQ22536 (100 μmol/L) and H89 (1 μmol/L), (B) compound C (CC; 10 μmol/L) and N^5-nitro-L-arginine methyl ester (l-NAME; 100 μmol/L, 30 minutes), or (C) by exendin 9-39 (Ex9-39; 100 nmol/L) and glucagon-like peptide 1 receptor (GLP-1R) antibody (GLP-1R Ab; 2.5 μg/mL, 2 hours). Control and exendin 4 groups are similar in A through C, D. Exendin 4 (10 nmol/L, 12 hours) had no effect on EDR in renal arteries from Wistar-Kyoto rats (WKYS). Data are mean±SEM. *P<0.05 vs control. #P<0.05 vs exendin 4. n=4 for WKYS; n=6 for SHRs.

Clinical and experimental studies suggest that GLP-1 and related agents in improving endothelial function in hypertension through the activation of AMPKα.

The actions of GLP-1R are thought to involve cAMP production and PKA activation.23,34 Kieffer and Habener35...
suggested the role of the GLP-1R and cAMP in the actions of GLP-1 on vascular endothelium. Moreover, PKA stimulates LKB1 for AMPK activation in hepatocytes. The present study demonstrated that sitagliptin stimulated the activation of LKB1/AMPK subsequent to cAMP/PKA signaling on activation of GLP-1R (Figure S10). Finally, the in vivo effect of sitagliptin was assessed in SHRs by measuring RBF and blood pressure. Chronic GLP-1 treatment lowers blood pressure in patients with type 2 diabetes mellitus, and exendin 4 also exerts an antihypertensive effect in salt-sensitive hypertensive mice. Another study suggests that DPP-4 inhibition by sitagliptin attenuates blood pressure elevation in SHRs, which may in part contribute to the blood pressure–lowering effect of sitagliptin.

**Perspectives**

We demonstrate that DPP-4 inhibition by sitagliptin preserves vascular GLP-1/GLP-1R function in SHRs, a genetic model of hypertension. GLP-1–induced AMPK/eNOS activation restores endothelium-dependent relaxation and RBF, thus helping to reduce SBP in SHRs. Like sitagliptin, the GLP-1R agonist exendin 4 is also effective in augmenting endothelial function in hypertensive rats and patients, thereby elucidating the mechanism underlying the vascular benefits of GLP-1 and related agents. Taken together, the novel findings of the present study highlight the prospect for the use of GLP-1–elevating agents and GLP-1R agonists against vascular dysfunction in hypertension.

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Disclosures
None.

References


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### Novelty and Significance

**What Is New?**

- Treatment with the dipeptidyl peptidase 4 inhibitor sitagliptin and GLP-1 receptor agonist exendin 4 improves endothelial function.
- Sitagliptin treatment lowers SBP and enhances RBF in spontaneously hypertensive rats.
- Sitagliptin treatment increases vascular GLP-1 receptor expression.
- Exendin 4 stimulates NO production and improves endothelial function in hypertensive patients.

**What Is Relevant?**

- The novel findings of the present study highlight the prospect for the use of GLP-1–elevating agents and GLP-1 receptor agonists against vascular dysfunction in hypertension.

**Summary**

The upregulation of GLP-1 and related agents preserves endothelial function in hypertension by restoring NO bioavailability.
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Supplemental Materials

Expanded Materials and Methods

**Animals**

Male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs) were supplied by the Chinese University of Hong Kong (CUHK) Laboratory Animal Service Center. This investigation was approved by the CUHK Animal Experimentation Ethics Committee and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). SHRs (32–40 weeks old) and WKYs (32–40 weeks old) received sitagliptin (10 mg/kg/day by oral gavage) or vehicle for 2 weeks.

**Blood Pressure Measurement**

SHRs were surgically implanted with telemetric transmitters (TL11M2-C50-PXT, Data Sciences International, Minnesota, USA). The catheter of the implant was placed into the distal portion of the descending aorta. Rats were allowed to recover from surgery for 7 days, and then 24h ambulatory systolic blood pressures were measured by telemetry in conscious, unrestrained rats. Data were collected for 20 seconds every 20 min and used the 24-hour mean values for analysis. The ambulatory arterial pressures were measured at Day 0 and Day 14 after sitagliptin treatment in SHRs and WKYs.

For direct catheter measurement, vehicle and sitagliptin-treated WKY and SHR were anesthetized. Systolic blood pressure was measured by inserting a heparinized saline-filled PE-50 catheter into the left common carotid artery after an initial 15 min equilibration period. In addition, systolic blood pressure was also measured by the tail-cuff method before and after sitagliptin treatment. Blood pressure was calculated from the average of 5 successive recordings.

**Measurement of Renal Blood Flow by Magnetic Resonance Image (MRI) Acquisition Procedure**

MRI studies were performed using a 3T clinical whole-body imaging system (Achieva, Philips Healthcare, Best, Netherlands). MRI contrast agent was gadolinium-tetrazacyclododecanetetraacetic acid (Gd-DOTA) (Guerbet Group, Roissy CDG cedex, France). After anesthesia, rats were positioned supinely. The MRI acquisition of the rat urinary system included high resolution T2 weighted axial plane anatomical examination, high resolution T1 weighted coronal plane anatomical examination, and dynamic contrast enhanced examination in coronal plane. Axial anatomical examinations were acquired with the following parameters: multiple slice turbo spine echo sequence, repetition time (TR)/time to echo (TE)/flip angle= 2359 ms/120 ms/90°, field of view = 60 mm×81 mm×30 mm, the acquisition voxel size was 0.41 mm×0.41 mm×1.50 mm, and the reconstructed voxel size was 0.17 mm×0.17 mm×1.5 mm. Coronal anatomical examinations were acquired with the following parameters: three-dimensional (3D)
gradient echo sequence with fat suppression, TR/TE/flip angle= 4.4 ms/2.2 ms/10°, field of view = 80 mm× 80 mm×18 mm, the acquisition voxel size was 0.50 mm×0.50 mm×1.00 mm and the reconstructed voxel size was 0.28 mm×0.28 mm×0.50 mm. The contrast-enhanced examinations were acquired with the following parameters: 3D gradient echo sequence, TR/TE/flip angle= 6.8 ms/2.3 ms/35°, field of view = 80 mm×80 mm×12 mm, the acquisition voxel size was 0.61 mm×0.75 mm×3.00 mm and the reconstructed voxel size was 0.31 mm×0.31 mm×1.5 mm. MRI contrast agent was gadolinium-tetraazacyclododecanetetraacetic acid (Gd-DOTA) (Guerbet Group, Roissy CDG cedex, France). A dose of 0.075 mmol/kg was injected through tail vein as a rapid bolus in less than 1 sec after initial baseline 10 acquisitions and followed by a flush of 0.5 mL normal saline. Dynamic scan was stopped when the contrast agent was excreted and clearly visible in the bilateral ureters.

MRI Analysis
The reconstructed MR images were transferred to a radiological workstation (Extended Workspace, Philips, Best, Netherlands) for off-line analysis. Anatomical images were read by a radiologist with animal research experiences. For analysis of dynamic data, regions of interest (ROIs) were manually drawn over left and right kidneys. The ROIs of the renal cortex were drawn in all rats. These ROIs were used on the perfusion-weighted data to generate time signal intensity curves.

Intrarenal Artery Preparation
Rats were sacrificed by CO₂ suffocation and intrarenal arteries were removed and placed in ice-cold Krebs solution (mmol/L): 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, and 11 D-glucose. Arteries were cleaned of adhering tissue and cut into ring segments of 2 mm in length. Arteries from SHR were incubated for 12 hours in Dulbecco’s Modified Eagle’s Media (DMEM, Gibco, Grand Island, NY, USA) culture media with 10% fetal bovine serum (FBS, Gibco), 100 IU penicillin and 100 μg/mL streptomycin with or without sitagliptin or exendin-4. Rings were suspended in myograph (Danish Myo Technology, Aarhus, Denmark) for recording of changes in isometric tension.² ³ Briefly, two tungsten wires (40 μm in diameter) were inserted through the lumen and fixed to jaws of organ chamber. The organ chamber was filled with 5 mL Krebs solution and gassed by 95% O₂-5% CO₂ at 37°C (pH ~7.4). Each ring was stretched to 2.5 mN, an optimal tension, and then allowed to stabilize for 90 min before the start of each experiment.²

Functional Studies
Each ring was initially contracted by 60 mmol/L KCl. Endothelium-dependent relaxation (EDR) to acetylcholine (ACH, 0.003 to 10 μmol/L) while endothelium-independent relaxation to sodium nitroprusside (SNP, 0.001 to 10 μmol/L) were examined in arteries pre-contracted with phenylephrine (1 μmol/L). In the first set of experiments, SHR renal arteries were incubated with exendin-4 (10 nmol/L, GLP-1 receptor agonist) for 12 hours before vasoreactivity study on wire myograph. In some experiments, GLP-1 receptor antibody (2.5 μg/mL) was added 2 hours before incubation with exendin-4 or incubation with SQ22536 (100 μmol/L, adenylyl cyclase inhibitor), H89 (1 μmol/L, PKA inhibitor), exendin 9-39 (100 nmol/L, GLP-1 receptor antagonist) and compound C (10 μmol/L, AMPKα inhibitor) along with exendin-4. Some arterial rings were subjected to 30-min exposure to L-NAME (100 μmol/L, nitric oxide synthase inhibitor) and then endothelium-dependent relaxations in response to cumulative additions of ACh were measured. The second series of experiments examined the impact of oral treatment with sitagliptin on endothelial function in SHRs. The relaxations to ACh in renal arteries from sitagliptin-treated SHRs were studied in control and in the presence of each of the following inhibitors (30-min incubation): SQ22536 (100 μmol/L), H89 (1 μmol/L), exendin 9-39 (100 nmol/L),⁴ compound C (10 μmol/L),⁵ or L-NAME (100 μmol/L).

Measurement of GLP-1 in Plasma
Plasma was kept from vehicle and sitagliptin-treated WKYs and SHRs. GLP-1 levels in plasma were assayed by Glucagon-Like Peptide-1 (Active) ELISA kit (Linco Research) according to the manufacturer’s instructions.

cAMP Levels in Renal Arteries
Renal arteries from sitagliptin-treated SHR were cultured with or without inhibitors and were prepared according to the manufacturer’s instructions. cAMP levels were assayed by Direct cAMP ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA).

Primary Culture of Rat Aortic Endothelial Cells
Aortas of SHR were dissected in sterilized phosphate buffered saline (PBS) under a stereoscopic microscope. After digestion by 0.2% collagenase for 15 minutes at 37°C, RPMI-1640 (Gibco) was added and endothelial cells were then collected by centrifugation at 1000 rpm for 5 minutes. Thereafter, the pellet was gently re-suspended in RPMI-1640 supplemented with 10% FBS and cultured in a 75-cm² cell culture flask. To remove other cell types, the medium was changed after 1-hour incubation, then maintained until 70% confluence before use.

Transfection Condition
SHR aortic endothelial cells were transfected with either a wild type AMPKα2 plasmid (WT-AMPK), a
dominant negative AMPK construct K45R (DN-AMPK), or control vector by electroporation using Nucleofector II machine (Amaxa/Lonza, Walkersville, MD, USA) according to the manufacturer’s instruction. About 70% of endothelial cells were successfully transfected as indicated by control transfection using a GFP-expressing pCAGGS vector.

Western Blot Analysis
Isolated renal arteries or SHR aortic endothelial cells were homogenized in RIPA lysis buffer that contained 1 µg/mL leupeptin, 5 µg/mL aprotinin, 100 µg/mL PMSF, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, and 2 µg/mL β-glycerophosphate, and centrifuged at 20,000 xg for 20 min at 4°C. Protein lysates (25 µg for arteries, 10 µg for cells) were separated by electrophoresis and transferred onto PVDF membrane. Blots were blocked with 1% bovine serum albumin or 5% non-fat milk for 1 hour and incubated overnight at 4°C with antibodies against phospho-PKA C (catalytic subunit, Thr197), phospho-LKB1 (Ser334), phospho-eNOS (Ser1177), phospho-AMPKα (Thr172), PKA Cα, LKB1, eNOS, AMPKα and AMPKα2, GLP-1 receptor and against mouse GLP-1 and GAPDH. After washing, blots were incubated with HRP-conjugated swine anti-rabbit or anti-mouse IgG. Immunoreactive bands were visualized by chemiluminescence and exposed to Kodak Image Station 440 for densitometric analysis.

Nitric Oxide (NO) Measurement
Endothelial cells seeded on glass coverslips were loaded with 1 µmol/L DAF-FM diacetate (Molecular Probes, Eugene, OR, USA) at room temperature for 10 minutes and placed in a designed chamber for fluorescence imaging. Intracellular NO production was monitored using a fluorescent NO indicator DAF-FM diacetate as described. 6 DAF-FM diacetate is cell-permeant and passively diffuses across cellular membrane. The fluorescence quantum yield of DAF-FM is ~0.005, but increases ~160 fold to ~0.81, after reacting with NO, which was measured by a confocal scanning unit (FV1000, Olympus, Tokyo, Japan) at excitation 488 nm and an emission filter of 505-525 nm. Changes in [NO] were displayed as a ratio of fluorescence relative to the intensity (F/F0), and analyzed by the Fluoview software (Olympus).

Total NO Production in Endothelial Cells
SHR endothelial cells were incubated in the presence of exendin-4 (10 nmol/L, 30min) with or without inhibitors. Total NO production in SHR endothelial cells was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, by the Total Nitric Oxide Assay Kit (Beyotime Biotechnology) according to the manufacturer’s instructions.

cGMP Levels in Renal Arteries
SHR renal arteries were cultured with sitagliptin (10 µmol/L) or exendin-4 (10 nmol/L) for 12 hours. The tissue were then frozen and stored at -80 °C until assay. The levels of cyclic GMP were measured by direct cGMP ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer’s instruction. The result was expressed as cyclic GMP production in pmol per mg protein.

Human Artery Specimen
The present study was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. Human renal arteries were obtained after informed consent from normotensive and hypertensive patients undergoing nephrectomy at ages between 50-80 years old. The indications for surgery included tumor (4 in normotensive patients and 3 in hypertensive patients) and poorly functioning kidney (2 in normotensive patients and 1 in hypertensive patients). History of hypertension was defined as having persistent elevated blood pressure, systolic blood pressure of >140 mm Hg, or diastolic blood pressure of >90 mm Hg and requiring medical therapy.

Materials and Drugs
Anti-phospho-eNOS (Ser1177), anti-eNOS, anti-GLP-1 receptor and anti-GLP-1 antibodies were obtained from Abcam (Cambridge, MA), Anti-phospho-PKA C (Thr197), phospho-LKB1 (Ser334), phospho-AMPKα (Thr172), anti-AMPKα, anti-PKA Cα, anti-LKB1 and anti-AMPKα2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GAPDH were obtained from Ambion (Austin, TX, USA). HRP-conjugated swine anti-rabbit or anti-mouse IgG were from DakoCytomation (Carpinteria, CA, USA). Immobilon-P polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, MA, USA) and chemiluminescence (ECL reagents) was obtained from Amersham Pharmacia. Phenylephrine, acetylcholine, L-NAME, sodium nitroprusside, H89, compound C, exendin-4, exendin 9-39 were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). SQ22536 was from Calbiochem (San Diego, CA, USA). The cell culture media and DAF-FM diacetate were from Invitrogen (Carlsbad, CA, USA). Sitagliptin was a kind gift from Merck Research Laboratories (Rahway, NJ, USA). SQ22536 and compound C were dissolved in DMSO and other drugs in distilled water. DMSO (0.1% v/v) did not modify agonist-induced responses.

Data Analysis
Results represent means±SEM from different animals. Concentration-response curves were analyzed by non-linear curve fitting using GraphPad Prism software (Version 4.0, San Diego, CA, USA). The negative logarithm of the dilator concentration that produced half of the maximum effect (pD2) and
the maximum relaxation ($E_{\text{max}}\%$) were calculated. The protein expression was quantified by densitometer (FluorChem, Alpha Innotech, San Leandro, CA, USA), and analyzed by Quantity One software (Bio-Rad). Statistical significance was determined by two-tailed Student’s t-test or one-way ANOVA followed by the Bonferroni post-hoc test when more than two treatments were compared. $p<0.05$ indicates statistically significant difference.

Additional Results
Telemetry results showed that systolic blood pressure (SBP) gradually reduced about one week after sitagliptin treatment in SHRs comparing to that before treatment or that of SHR treated with vehicle at the same time. Mean arterial pressure (MBP) also reduced in SHRs after sitagliptin treatment comparing to that before treatment. Diastolic BPs were similar in all the groups. No obvious diurnal rhythm of BP or HR was found in all the groups of rats (Supplemental Fig. S1C).

Additional Discussion
However, the effect of DPP-4 inhibitors on arterial blood pressure is still disputed. DPP-4 inhibition has no and can either lower or elevate blood pressure depending on types of inhibitors, the duration of treatment, and the level of background anti-hypertensive treatment.7, 8 This variation in the impact on blood pressure is likely related to the fact that multiple substrates are catalyzed by DPP-4. Thus, the advantages and disadvantages of the use of DPP-4 inhibitors with respect to the cardiovascular function can be influenced by the relative amount of various bioactive substrates such as vasoconstrictive neuropeptide Y, their action on sympathetic nerves, and hemodynamic interaction with other blood pressure regulators such as angiotensin-converting enzyme.9 In this study, we only observed reduction of systolic blood pressure during 14-day treatment with sitagliptin as measured by telemetry, without significant change of diastolic and mean arterial pressure. Further detailed study on blood pressure regulation by DPP-4 inhibitor may be useful to provide more information on blood pressure control in different animals under normotensive or hypertensive conditions.

Limitations of the present study
The present study demonstrates that DPP-4 inhibition improves endothelium-dependent relaxations in renal interlobar arteries of SHR. The effect of DPP-4 inhibition is not examined in resistance arteries which also contribute to the regulation of blood pressure. In addition, whether DPP-4 inhibition also decreases vascular inflammation or whether it improves renal function through other mechanisms that are associated with blood pressure regulation are not studied. There are other methods which can measure the vasomotor response of renal arterioles,10, 11 which are more important for blood pressure regulation, so this method will be employed to observe the response of arterioles to GLP-1 in the future investigations. However, this is beyond the scope of the present study.

References
10. Al-Mashhadi RH, Skott O, Vanhoutte PM, Hansen PB. Activation of A(2) adenosine

Figure S1. Systolic blood pressure (SBP) of rats. SBP were measured by direct measurement of SBP with a direct catheter in anesthetized rats (A) and the tail-cuff method (B). Two-week oral administration with sitagliptin (10 mg/kg/day) decreased SBP of SHRs without affecting the SBP of WKYs. (C) The ambulatory systolic (SBP), diastolic (DBP), and mean arterial (MABP) pressures and heart rates (HR) were shown in sitagliptin-treated SHRs and WKYs compared to the vehicle-treated controls. Data are means±SEM. *p<0.05 vs WKY vehicle, #p<0.05 vs SHR vehicle. n=4.
Figure S2. Levels of GLP-1 and GLP-1 receptor in rats. Two-week oral administration of sitagliptin (10 mg/kg/day) increased the plasma concentration of GLP-1 (A), and GLP-1 (B) and GLP-1 receptor (C) expressions in renal arteries from WKY and SHRs. Data are means ± SEM. *p<0.05 vs WKY vehicle, #p<0.05 vs SHR vehicle. n=6 for Western blotting; n=8 for WKY ELISA; n=10 for SHR ELISA.

Figure S3. Levels of cAMP in SHR renal arteries. Chronic sitagliptin treatment increased cAMP levels in SHR renal arteries, which were inhibited by 30 min-incubation with exendin 9-39 (100 nmol/L) and SQ22536 (100 μmol/L) but not by compound C (10 μmol/L). Data are means±SEM. *p<0.05 vs vehicle, #p<0.05 vs sitagliptin. n=6.
Figure S4. Exendin-4 stimulates NO production in SHR aortic endothelial cells. Inhibitory effects of exendin 9-39 (Ex9-39, 100 nmol/L) (A), SQ22536 (100 µmol/L) and H89 (1 µmol/L) (B), compound C (CC, 10 µmol/L) and L-NAME (100 µmol/L) (C) on NO production stimulated by exendin-4 (ex4). (D) Over-expression of AMPKα2 (WT) further elevated NO production stimulated by exendin-4 and these effects were inhibited by suppression of AMPK activity through expression of DN-AMPK (DN). (E) Images showing NO production under various treatments. Data are means±SEM. *p<0.05 vs control. #p<0.05 vs exendin-4. n=6 for control; n=4 for various treatments.
Figure S5. Total NO production in SHR aortic endothelial cells. Total NO production in SHR endothelial cells was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, by modified Griess reaction method. Exendin-4 (10 nmol/L, 30min) increased NO production, which was inhibited by exendin 9-39 (100 nmol/L), SQ22536 (100 µmol/L), H89 (1 µmol/L), or compound C (10 µmol/L). Data are means±SEM. *p<0.05 vs control. #p<0.05 vs exendin-4. n=5.

Figure S6. cGMP levels in SHR renal arteries. Incubation with sitagliptin (10 µmol/L, 12 hours) or exendin-4 (10 nmol/L, 12 hours) increased cGMP levels in SHR renal arteries. Data are means±SEM. *p<0.05 vs vehicle. n=4.
Figure S7. Exendin-4 increases AMPKα and eNOS phosphorylations in SHR aortic endothelial cells. Overexpression of AMPKα2 (WT-AMPK, WT) further increased phosphorylations of AMPKα (A) and eNOS (B) stimulated by exendin-4 (10 nmol/L, 12 hours) in SHR endothelial cells, while expression of DN-AMPK (DN) inhibited these effects. Data are means±SEM. *p<0.05 vs control (C). #p<0.05 vs exendin-4. n=6.

Figure S8. Levels of AMPKα2 in cultured SHR aortic endothelial cells. The level of AMPKα2 increased by expression of WT-AMPK (WT) and unaffected by expression of DN-AMPK (DN) compared with control (C, Cont). Data are means±SEM. *p<0.05 vs control. #p<0.05 vs WT. n=4.
Figure S9. Insulin tolerance test (ITT) of rats. There was no difference in ITT between WKY and SHR at the age used in the present study. Sitagliptin had no effect on ITT in SHR. Data are means ± SEM. n=4.

Figure S10. The proposed cellular mechanism for the protective effect of sitagliptin against endothelial dysfunction in hypertension. The bioavailability of NO decreased in hypertension. Sitagliptin enhances phosphorylation of eNOS at Ser1177 via AMPKα phosphorylation at Thr172 through the activation of GLP-1R/cAMP/PKA/LKB1 cascade, leading to elevated NO level and thus improves endothelial function in hypertension.