Endothelial Function

Activation of Transient Receptor Potential Channel Vanilloid 4 by DPP-4 (Dipeptidyl Peptidase-4) Inhibitor Vildagliptin Protects Against Diabetic Endothelial Dysfunction

Peng Gao*, Li Li*, Xiao Wei, Miao Wang, Yangning Hong, Hao Wu, Yanjia Shen, Tianyi Ma, Xing Wei, Qin Zhang, Xia Fang, Lijuan Wang, Zhencheng Yan, Guan-Hua Du, Hongting Zheng, Gangyi Yang, Daoyan Liu, Zhiming Zhu

Abstract—Endothelial dysfunction is an early step to the progression of cardiovascular diseases in diabetes. Apart from their anti-diabetic action, DPP-4 (dipeptidyl peptidase-4) inhibitors also reduce cardiovascular events in diabetic patients. However, the underlying mechanism of the beneficial effect of DPP-4 inhibitor on endothelial function is still obscure. In this study, we intervened type 1 or 2 diabetic model mice with vildagliptin for 4 weeks and measured the vascular reactivity. We found that vildagliptin improved endothelium-dependent vasodilation in diabetic mice independent of GLP-1 (glucagon-like peptide-1), but this effect was blocked by a SIRT1 (Sir2 homolog 1) inhibitor, Ex527. Mechanistically, vildagliptin-activated Transient Receptor Potential Channel Vanilloid 4 (TRPV4) to promote extracellular calcium uptake in endothelial cells, which activated AMPK (AMP-activated protein kinase)/SIRT1 pathway to counteract hyperglycemia-induced endothelial reactive oxygen species generation and senescence. Vildagliptin directly binds to TRPV4 by forming a hydrogen bond, which is critical to vildagliptin-evoked endothelial calcium intake. Knockout or inhibition of TRPV4 erased the beneficial role of vildagliptin. In addition, activation of SIRT1 by SRT1720 improved endothelial function independent of TRPV4 and reduced TRPV4 transcription to maintain an appropriate calcium level. In summary, our findings prove that vildagliptin protects against hyperglycemia-induced endothelial dysfunction by activating TRPV4-mediated Ca2+ uptake, which helps to re-understand the mechanism of DPP-4 inhibitors and expand the therapeutic scope. (Hypertension. 2020;75:150-162. DOI: 10.1161/HYPERTENSIONAHA.119.13778.)

Key Words: diabetes complications ▪ sirtuin 1 ▪ transient receptor potential channels ▪ vascular endothelial cells ▪ vildagliptin

Endothelial dysfunction, characterized by impaired endothelium-dependent vasorelaxation, is an early step and important contributor to the progression of cardiovascular diseases, diabetes mellitus, and the associated vasculopathies resulting in end organ damage.1 Hyperglycemia acts as an early trigger in the development of endothelial dysfunction as the progression of complications are delayed in diabetic patients with good glycemic control.2,3 Sustained hyperglycemia causes an increase in reactive oxygen species (ROS), which ultimately results in the onset of apoptosis or the induction of cellular senescence.4,5 Therefore, intervention aimed to protect against hyperglycemia-induced endothelial dysfunction is crucial for the prevention and improvement of diabetic vascular complications.

Endothelial cells regulate arterial tone by producing nitric oxide and prostacyclin, which diffuse to underlying smooth muscle cells to evoke vasodilation. This process is modulated by Ca2+.6 The Transient Receptor Potential (TRP) family of ion channels is the major class of Ca2+ permeable ion channels in the endothelium.6 Transient Receptor Potential Channel Vanilloid 4 (TRPV4) is among the most described ion channels.
flow-mediated dilation of arteries. TRPV4-mediated endothelial Ca²⁺ influx participates in Ach-induced vascular relaxation by increasing the release of nitric oxide. In addition, the expression of TRPV4 in endothelial cells could be suppressed by high glucose or hyperglycemia in diabetic mice, implying that the reduced functional TRPV4 might be involved in hyperglycemia-induced endothelial dysfunction.

The DPP-4 (dipeptidyl peptidase-4) inhibitors are extensively used antidabetic drugs by increasing circulating levels of GLP-1 (glucagonlike peptide-1), an incretin critical to glucose-stimulated insulin release. Experimental and clinical studies provide numeral evidence showing that DPP-4 inhibitors also exert potent effects to reduce cardiovascular events. Vildagliptin is the most thoroughly studied one among the corresponding antidiabetic drugs by increasing circulating levels of GLP-1 (glucagonlike peptide-1), an incretin critical to glucose-stimulated insulin release. 8,9 In addition, the regulatory effect of TRPV4 on SIRT1 that participate in the protective role of TRPV4 in hyperglycemia-induced endothelial dysfunction remains elusive.

The DPP-4 (dipeptidyl peptidase-4) inhibitors are extensively used antidabetic drugs by increasing circulating levels of GLP-1 (glucagonlike peptide-1), an incretin critical to glucose-stimulated insulin release. 8,9 In addition, the regulatory effect of TRPV4 on SIRT1 that participate in the protective role of TRPV4 in hyperglycemia-induced endothelial dysfunction remains elusive.

As the best characterized member of mammalian protein deacetylase sirtuins with a relatively high expression in the vasculature, SIRT1 acts as a powerful guardian antagonizing vascular endothelial dysfunction induced by various kinds of stimuli, including hyperglycemia. 18-21 Our previous study also proved that SIRT1 prevents vascular senescence and improves vascular function by downregulation of PAI-1 (plasminogen activator inhibitor-1), a senescence marker. 22 We also reported that apigenin, a TRPV4 activator, exerted an antifibrotic role in kidney by upregulating SIRT1. 23 Thus, there might exist a regulatory effect of TRPV4 on SIRT1 that participate in the beneficial role of TRPV4 in modulating endothelial function.

In the present study, we investigated the potential participation of TRPV4 in the protective effect of vildagliptin on hyperglycemia-induced endothelial dysfunction. We have determined that the beneficial role of vildagliptin is GLP-1 independent and mediated by activation of AMPK/SIRT1 pathway in endothelial cells.

**Methods**

The data that support the findings of this study are available from the corresponding author on reasonable request. The detailed experimental protocols are available in the online-only Data Supplement.

**Animals, Establishment of Diabetic Models and Drug Treatment**

The db/db mice (000697) were purchased from Jackson Laboratory (Bar Harbor, ME). TRPV4 knockout mice (TRPV4 −/−) were kindly provided by Professor Xin Ma from Jiangnan University, China. All mice were housed in cages at a controlled temperature (22±1 °C) and relative humidity (55±5%) in a 12-h light/12-h dark cycle. They were supplied with standard laboratory chow and tap water ad libitum. All experimental procedures were performed in accordance with protocols approved by the institutional animal care and research advisory committee at Daping hospital, Third Military Medical University.

Six-week-old male C57BL/6 mice and TRPV4 −/− mice, weighing 18 to 22g, were randomly divided into 2 groups. One group was made diabetic by peritoneal injection of streptozotocin as previously described. 20 Drug treatment was not performed until 4 months of age when all diabetic mice, including db/db mice, displayed obvious diabetic features. The vildagliptin powder was provided by Novartis Pharma AG (Switzerland). Vildagliptin was administered (50 mg/kg body weight) once daily by oral gavage for 4 weeks, the control group received same amount of water as vehicle. Exendin 9–39 (E7269, Sigma-Aldrich) was delivered by subcutaneously implanted Alzet miniosmotic pumps (model 2002; Alza, Palo Alto, CA) at a rate of 150 pmol/kg/minute for 2 weeks before end of the experiment as described. 22 Ex527 (E7034) was purchased from Sigma-Aldrich, dissolved in 4% DMSO/10% cyclodextrin in PBS, and administered intraperitoneally at a concentration of 1 mg/kg body weight daily for 10 days before the mice were sacrificed as previously described. 22 In some experiments, mice were treated with 100 mg/kg body weight SRT1720 (A4180, Apexbio) or vehicle (40% PEG-400/0.5% Tween-80/59.5% deionized water) for 4 weeks via oral gavage. 22 Systolic blood pressure (SBP) and blood glucose were measured every week. At the end of experiments, plasma GLP-1, PAI-1, and urine 8-hydroxy-2′-deoxyguanosine were measured. And the thoracic aortas were removed and the vascular reactivity was measured as previously described. 22 Some aortas were removed and stored at −70°C for western blot or real-time PCR analysis. The primers used in real-time PCR analysis are listed in Table S1 in the online-only Data Supplement.

**Results**

**Vildagliptin Improved Endothelial-Dependent Vasorelaxation Independent of GLP-1**

Two types of diabetic models were used to investigate the impacts of vildagliptin on the physiological and biochemical parameters in mice. Before drug intervention, the body weight of streptozotocin-induced type 1 diabetic mice decreased significantly and their fasting blood glucose levels (FBG) maintained at a high level (Figure 1A), whereas both body weight and FBG were remarkably increased in type 2 diabetic db/db mice (Figure S1A). After 4-week treatment, vildagliptin failed to ameliorate either lowered body weight or elevated FBG level in type 1 diabetic mice (Figure 1A) but slowed down the rapid body weight gain in db/db mice without affecting their FBG (Figure S1A). In addition, vildagliptin did not show any beneficial effect on glucose tolerance of streptozotocin-injected mice (Figure 1B), although there was a relatively higher serum level of GLP-1 (Figure 1C). However, vildagliptin slightly improved glucose tolerance in db/db mice, with a decreased AUC and higher serum GLP-1 level (Figure S1B and S1C). In streptozotocin-injected mice or db/db mice, there was no obvious alteration in heart rate or SBP, and vildagliptin did not show any obvious effects (Figure 1D; Figure S1D).

To exclude the influence of blood glucose level or glucose tolerance on vascular reactivity, streptozotocin-induced type 1 diabetic mice were chosen in isometric tension studies to evaluate the effect of vildagliptin on endothelium-dependent vasorelaxation (EDR). EDR to acetylcholine (Ach) was significantly improved in vildagliptin-treated diabetic mice compared with placebo.
Hypertension January 2020

Figure 1. Vildagliptin fails to affect the general physiological parameters but improves endothelium-dependent vasodilation in streptozotocin-induced diabetic mice. A, Changes of body weight (left) and fasting blood glucose (right) in vildagliptin or vehicle-treated mice injected with streptozotocin or vehicle (n=8). B, Blood glucose levels during intraperitoneal glucose tolerance test (2 g/kg) performed on vildagliptin or vehicle-treated mice injected with streptozotocin or vehicle (n=8). The areas under curve of each group are shown on the right. C, The serum GLP-1 levels of vildagliptin or vehicle-treated mice injected with streptozotocin or vehicle (n=8). D, Changes of heart rate and systolic blood pressure in vildagliptin or vehicle-treated mice injected with streptozotocin or vehicle during experimental process (n=8). E, Top: the phenylephrine (PE)-induced contraction, acetylcholine (Ach), or sodium nitroprusside (SNP)-induced relaxation of the isolated aortas from vildagliptin or vehicle-treated mice injected with streptozotocin or vehicle (n=8). Bottom: The effect of L-NAME or exendin 9-39 preincubation on Ach-induced vasorelaxation of the isolated aortas from indicated groups (n=8). F, The Ach-induced relaxation of the aortas from vildagliptin and/or exendin 9-39-treated mice injected with streptozotocin or vehicle (n=8). The data are presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001, compared between streptozotocin and vehicle-treated group; *P<0.05, **P<0.01, ***P<0.001, compared between vildagliptin and vehicle-treated group.
with controls (Figure 1E). And no difference was observed in vascular dilations in response to sodium nitroprusside or contractions in response to phenylephrine (Figure 1E). In addition, the promotional effect of vildagliptin on EDR was totally blocked by NG-nitro-l-arginine methyl ester, a nitric oxide chelator (Figure 1E). In addition, the inhibitor of GLP-1 receptor, exendin 9-39, failed to block the improvement of EDR in the aortas from vildagliptin-treated diabetic mice, either directly incubating isolated blood vessels or 2-week in vivo intervention (Figure 1E and 1F), suggesting that promotional effect of vildagliptin on EDR was not dependent on GLP-1.

Vildagliptin Stimulates SIRT1 Expression by Increasing the Intracellular Calcium Level

As AMPK serves as an upstream regulator of SIRT1, to further investigate the detailed mechanism of how vildagliptin regulates the expression of SIRT1, the influence of an AMPK inhibitor, compound C, on the beneficial role of vildagliptin was assessed in HUVECs. High glucose significantly reduced the phosphorylation of AMPK (Thr172), which was restored by vildagliptin (Figure 3A and 3B). Importantly, treatment of compound C not only reduced the protein level of phosphorylated AMPK in vildagliptin-treated HUVECs but also almost totally blocked the promotional effect of vildagliptin on the expression of SIRT1, accompanied by an upregulation of TNF-α, NOX1 and PAI-1 (Figure 3A and 3B). Moreover, inhibition of AMPK by compound C also lowered the NAD+/NADH ratio, a main index stimulating SIRT1 activity, in vildagliptin-treated HUVECs (Figure S4A and S4B). Knockdown of the AMPK upstream kinase serine/threonine kinase 11 by siRNA did not obviously affect the promotional effect of vildagliptin on the phosphorylation of AMPK in high glucose-treated HUVECs, but knockdown of another Ca2+-sensitive upstream regulator Ca2+/calmodulin-dependent protein kinase β abolished the response of AMPK phosphorylation to vildagliptin (Figure 3C and 3D). In consistence, treatment of HUVECs with STO-609, a Ca2+/calmodulin-dependent protein kinase β inhibitor, or directly blocking the intracellular Ca2+ using BAPTA-AM, a Ca2+ chelator, all significantly inhibited the promotional effect of vildagliptin on the phosphorylation of AMPK, resulting in a lowered SIRT1 expression in high glucose–treated HUVECs (Figure 3E and 3F). These results indicate that vildagliptin promotes phosphorylation of AMPK and SIRT1 expression in an intracellular Ca2+-dependent manner.

Vildagliptin Normalizes Hyperglycemia-Reduced Intracellular Ca2+ Level and Acts As An Activator of TRPV4

To directly evaluate the impact of vildagliptin on intracellular Ca2+ level in HUVECs, the ability of extracellular Ca2+ uptake was monitored. As expected, treatment of high d-glucose remarkably decreased the basal Ca2+ level and Ca2+ uptake in HUVECs, whereas vildagliptin restored the impaired Ca2+ uptake ability and normalized Ca2+ influx to a normal level (Figure 4A; Figure S5A). As a Ca2+ permeable channel, TRPV4 channels are also expressed on the vascular endothelium. Thus, we suspected that TRPV4 might participate in the beneficial role of vildagliptin. Indeed, both mRNA and protein levels of TRPV4 were significantly reduced in aortas of 2 diabetic model mice, which were obviously elevated by treatment of vildagliptin (Figure 4B; Figure S5B). In consistence, the expression of TRPV4 also displayed a similar trend in response to high glucose and vildagliptin in HUVECs (Figure 4C; Figure S5C). Next, we performed molecular docking using software to determine the potential binding of vildagliptin on TRPV4 protein. The results showed that all 3 TRPV4 activators, including vildagliptin, positive control GSK1016790A, and a previous reported TRPV4 activator apigenin, can form hydrogen bonds with the lysine 192 residue of the crystal, that is the lysine 340 of TRPV4 protein, whereas the hydrogen bond did not exist in TRPV1 (Figure 4D; Figure S6). None of other DPP-4 inhibitors, including linagliptin, saxagliptin, sitagliptin, and teneligliptin, could form the hydrogen bond with lysine 340 residue (Figure S7). Thus, to determine the importance of the hydrogen bond in the effect of vildagliptin, we constructed the mutant expression plasmid at this site and transfected it into Hela cells, which does not express endogenous TRPV4 protein. We found that compared with wild-type plasmid, mutation of the lysine 340 to glycine significantly inhibited vildagliptin-induced Ca2+ influx and single-channel currents, while mutation of another adjacent leucine 348, the leucine
Figure 2. The protective effects of vildagliptin on endothelial function is SIRT1 (sirtuin 1) dependent. A, Representative images of reactive oxygen species generation of human umbilical vein endothelial cells treated with high glucose, vildagliptin, and Ex527, detected by dihydroethidium staining. Bar represents 100 μm. The quantitative results are shown on the right (n=10). B, Representative images of senescence-associated β-gal staining of human umbilical vein endothelial cells treated with high glucose, vildagliptin, and Ex527. Bar represents 200 μm. The quantitative results are shown on the right (n=9). C, The serum PAI-1 and urinary 8-hydroxy-2′-deoxyguanosine levels of vildagliptin or vehicle-treated mice injected with streptozotocin or vehicle (n=8). D, Representative western blots of indicated genes in the aortas of vehicle or vildagliptin-treated mice injected with streptozotocin or vehicle. E, Representative western blots of indicated genes in human umbilical vein endothelial cells treated by high glucose, vildagliptin, and Ex527. GAPDH served as a loading control. F, The relaxation induced by acetylcholine (Ach) of the isolated aortas from vildagliptin or vildagliptin plus Ex527-treated mice injected with streptozotocin (n=8). The data are presented as the means±SD. *P<0.05, **P<0.01, compared between D-glucose and L-glucose (A–C) or streptozotocin+vildagliptin group (F); #P<0.05, ##P<0.01, ###P<0.001, compared between vildagliptin and vehicle-treated group. $$$P<0.001, compared between Ex527 and vehicle-treated group (A and B).
Figure 3. Vildagliptin activates AMPK (AMP-activated protein kinase)/SIRT1 (sirtuin 1) pathway in human umbilical vein endothelial cells in a Ca²⁺-dependent manner. A and B, Representative western blots of indicated genes in human umbilical vein endothelial cells treated by high glucose, vildagliptin, and compound C. The quantitative results are shown in B; (n=3). C and D, Representative western blots of indicated genes in human umbilical vein endothelial cells treated by high glucose, vildagliptin, serine/threonine kinase 11, and si-Ca²⁺/calmodulin-dependent protein kinase β. The quantitative results are shown in D; (n=3). E and F, Representative western blots of indicated genes in human umbilical vein endothelial cells treated by high glucose, vildagliptin, STO-609, and B acetoxymethyl-1, 2-bis [2-aminophenoxy] ethane-N, N', N', N'-tetracetic acid. The quantitative results are shown in F; (n=3). Results shown are representative of 3 blots. GAPDH served as a loading control. The data are presented as the mean±SD. *P<0.05, **P<0.01, ***P<0.001, compared between d-glucose and l-glucose; ##P<0.01, ###P<0.001, compared between vildagliptin and vehicle-treated group. $$$P<0.01, $$$$P<0.001, compared between other treatments and their corresponding control group.
200 in the crystal, displayed no such effect (Figure 4E; Figure S5D). These results suggest that vildagliptin activates TRPV4-mediated Ca\textsuperscript{2+} influx by forming hydrogen bonds with lysine 340 of TRPV4 protein. Moreover, the patch-clamp analysis revealed a conductance of inward and outward currents in response to vildagliptin treatment.
in a cell-attached model, which was significantly reduced by addition of HC067047, a TRPV4 antagonist (Figure 4F; Figure S5E). Consistently, vildagliptin also stimulated a remarkable Ca\(^{2+}\) influx in HUVECs cultured in normal media, which was almost totally blocked by HC067047 (Figure S5F). These results indicate that vildagliptin functions as a direct activator of TRPV4 in HUVECs.

**TRPV4 Mediates the Inhibitory Effect of Vildagliptin on Hyperglycemia-Induced Endothelial Dysfunction**

Then, we evaluated the contribution of TRPV4 in the beneficial effect of vildagliptin both in vitro and in vivo. In HUVECs, vildagliptin increased intracellular Ca\(^{2+}\) level in HUVECs treated with d-glucose, and this response was largely abolished using siRNA targeted against TRPV4 (Figure 5A), HC067047 (Figure 5B), or removal of extracellular Ca\(^{2+}\) using EDTA (Figure S8A), indicating that the promotional effect of vildagliptin on increasing intracellular Ca\(^{2+}\) level was dependent on TRPV4-mediated influx of extracellular Ca\(^{2+}\). Capsazepine, a TRPV1 antagonist, did not show such effect (Figure S8B). In the presence of cyclopiazonic acid, an agent used to empty Ca\(^{2+}\)-stores, vildagliptin still produced a remarkable increase in Ca\(^{2+}\) uptake after exogenous Ca\(^{2+}\) was added (Figure S8C). Knockdown of TRPV4 also erased the inhibitory effect of vildagliptin on high glucose–induced ROS generation (Figure 5C). Accordingly, treatment of si-TRPV4 or HC067047 not only elevated the expression levels of TNF-α, NOX1, and PAI-1 to counteract vildagliptin but also blocked the promotional effect of vildagliptin on phosphorylation of AMPK and SIRT1 expression in HUVECs treated with high glucose (Figure 5D; Figure S9A). Next, we determined the critical role of TRPV4 in mediating the beneficial role of vildagliptin using TRPV4\(^{-/-}\) mice.\(^{24}\) As TRPV4 knockout protects against the development of type 2 diabetes mellitus,\(^{25}\) we established type 1 diabetic model on TRPV4\(^{-/-}\) mice to exclude the impact of basal blood glucose level. As expected, there was no significant difference of fasting blood glucose level between TRPV4\(^{-/-}\) mice and their littermate control (Figure S8D). Also, no difference of SBP or heart rate existed in TRPV4\(^{-/-}\) mice compared with TRPV4\(^{+/+}\) mice (Figure S8D). However, knockout of TRPV4 directly impaired Ach-induced vascular relaxation in phenylephrine-precontracted aortas and the protective effect of vildagliptin on EDR was remarkably blocked in those from TRPV4\(^{-/-}\) mice (Figure 5E). Consistently, the inhibitory effect of vildagliptin on streptozotocin-evoked expression of TNF-α, NOX1, and PAI-1 in the arterial wall was diminished in TRPV4\(^{-/-}\) mice, accompanied with reduced activation of AMPK and SIRT1 (Figure 5F; Figure S9B). These results prove that TRPV4 acts as a mediator of the beneficial effect of vildagliptin on hyperglycemia-induced endothelial dysfunction.

**Overexpression of SIRT1 Inhibits TRPV4 in a Feedback Fashion**

As SIRT1-endothelial specific transgene or activation of SIRT1 by SRT1720 lowered FBG without affecting SBP or heart rate in TRPV4\(^{-/-}\) mice injected with streptozotocin (Figure 6A; Figure S10A). And SRT1720-treated diabetic TRPV4\(^{-/-}\) mice also displayed an improved EDR compared with vehicle controls (Figure 6B). Correspondingly, SRT1720 also reduced streptozotocin-evoked arterial expression of TNF-α, NOX1, and PAI-1 in TRPV4\(^{-/-}\) mice by activation of SIRT1 (Figure 6C; Figure S10B). These results indicate that SIRT1 acts as a downstream target of TRPV4 to protect against endothelial dysfunction. In addition, adenovirus-mediated overexpression of SIRT1 or treatment of SRT1720 obviously attenuated high glucose-augmented expression of TNF-α, NOX1, and PAI-1 in HUVECs treated with d-glucose (Figure S11A and S11B). A similar trend was found in HUVECs treated by SRT1720 (Figure S11C and S11D). In contrast, the SIRT1 inhibitor, Ex527, significantly enhanced vildagliptin-induced TRPV4 expression in HUVECs treated with l-glucose, but not d-glucose (Figure 6F; Figure S10D). To further investigate the inhibitory effect of SIRT1 on the expression of TRPV4, we screened the TRPV4 gene and identified potential AP-1-binding sites on the TRPV4 regulatory elements binding region. Through ChIP assay, we observed that treatment with l-glucose or d-glucose did not affect the AP-1 binding level on TRPV4 promoter (Figure S12A), indicating that the inhibitory effect of high glucose on TRPV4 expression was not AP-1 dependent. However, vildagliptin significantly increased the AP-1 binding level on the promoter of TRPV4 in HUVECs treated with either l-glucose or d-glucose, which was almost totally blocked by BAPTA-AM, suggesting that there existed a Ca\(^{2+}\)-dependent positive feedback loop between vildagliptin and TRPV4 expression (Figure S12B). Importantly, Ad-SIRT1 or SRT1720 directly reduced AP-1 binding by increasing the recruitment of SIRT1 on the promoter of TRPV4 (Figure S12C and S12D). These results suggest that there also exists a negative feedback regulation between SIRT1 and TRPV4 possibly to maintain an appropriate intracellular Ca\(^{2+}\) level by restricting the transcription of TRPV4.

**Discussion**

The present study demonstrates that DPP-4 inhibitor vildagliptin protects against hyperglycemia-induced endothelial dysfunction in a GLP-1-independent manner. Mechanistically, vildagliptin acts as a direct activator of TRPV4 by binding to a pocket in its fifth ankyrin repeat domain. Vildagliptin-induced Ca\(^{2+}\) uptake increased phosphorylation of AMPK and activates SIRT1, a key molecular suppressing excessive ROS generation and cellular senescence. Moreover, SIRT1 reversely reduces the transcription of TRPV4 by inhibiting AP-1 binding on its promoter to maintain an intracellular Ca\(^{2+}\) homeostasis. These findings have clarified how vildagliptin improves endothelial-dependent vasorelaxation and identified TRPV4 as a novel target of vildagliptin.
Figure 5. Transient Receptor Potential Channel Vanilloid 4 (TRPV4) mediates the protective effects of vildagliptin against endothelial dysfunction. A and B, Thapsigargin-induced SR Ca\(^{2+}\) release and Ca\(^{2+}\) uptake via store-operated channels (SOCs) after SR Ca\(^{2+}\) depletion in c-glucose–pretreated human umbilical vein endothelial cells preincubated with si-TRPV4 (A) or HC067047 (B). Cells were incubated in a Ca\(^{2+}\)-free buffer in the presence of Fura-2 (1 \(\mu\)mol/L). Peak \(F/F_0\) following thapsigargin (1 mmol/L) stimulation and after reintroducing 100 \(\mu\)mol/L extracellular Ca\(^{2+}\). The quantitative results are shown on the right (n=3 independent experiments). The data in (A and B) are presented as the mean±SD. *P<0.05, ***P<0.001, compared between vildagliptin group and other drugs; #P<0.05, ###P<0.001, compared between vildagliptin and vehicle groups. C, The quantitative results of reactive oxygen species (ROS) generation in HUVECs treated with si-TRPV4 and vildagliptin, detected by DHE staining (n=10). *P<0.05, ***P<0.001, compared with si-con group; ###P<0.001, compared with vehicle. D, Representative western blots of indicated genes in human umbilical vein endothelial cells treated by high glucose, vildagliptin, si-TRPV4 and/or HC067047. Results shown are representative of three blots. E, The relaxation induced by acetylcholine (Ach) or phenylephrine (PE)–induced contraction of the isolated aortas from streptozotocin-induced diabetic TRPV4\(^{+/+}\) or TRPV4\(^{-/-}\) mice treated with or without vildagliptin (n=8). *P<0.05, ***P<0.001, compared with TRPV4\(^{-/-}\) group; ###P<0.001, compared between vildagliptin and vehicle control. F, Representative Western Blots of indicated genes in the aortas of streptozotocin-induced diabetic TRPV4\(^{+/+}\) or TRPV4\(^{-/-}\) mice treated with or without vildagliptin. Results shown are representative of 3 blots.
Almost all TRP channels are permeable to Ca\(^{2+}\), and many of them display strong influence on Ca\(^{2+}\)-dependent signaling pathways by mediating direct Ca\(^{2+}\) influx, receptor-operated Ca\(^{2+}\) influx, or store-operated Ca\(^{2+}\) release.\(^6\) The critical role of TRPV4 in mediating flow or agonist-induced EDR by promoting extracellular Ca\(^{2+}\) influx has been well documented because the Ca\(^{2+}\) sparklets evoked by TRPV4 generate subcellular microdomains containing a very high Ca\(^{2+}\) level enough to activate a variety of Ca\(^{2+}\)-dependent signaling cascades.\(^{29}\) In accordance with our study, Gutterman and colleagues have confirmed that Ach-induced dilation was blunted in TRPV4\(^{-/-}\) mice with a decreased Ca\(^{2+}\) influx and nitric oxide production.\(^{9}\) However, we and others have confirmed that knockout or inhibition of TRPV4 does not affect basal blood pressure\(^{24,30}\) but facilitates blood pressure increase in response to other stimuli.\(^{31}\) Similar to a previous report,\(^{10}\) we also observed a decreased expression level of TRPV4 in aortas of both 2 diabetic model mice.
suggested that hyperglycemia is an important factor for the reduced TRPV4 expression, and impairs the endothelial-dependent relaxation in diabetic mice.

Although vildagliptin lowers risk of major adverse cardiac events in patients with type 2 diabetes mellitus, the detailed mechanism remains elusive. Most studies indicate that the protective role of vildagliptin in endothelial injury or senescence is related to the increased GLP-1 level. Here, we observed that vildagliptin improved EDR with the inhibitor of GLP-1 receptor and vildagliptin also directed the high glucose-induced ROS generation and senescence in absence of GLP-1, further suggesting a GLP-1-independent mechanism. Based on the fact that the increase of intracellular Ca²⁺ level was fundamental to the protective effect of vildagliptin, we discovered a direct binding site of vildagliptin in TRPV4 protein. As ATP acts as a ligand of TRPV4 to stimulate extracellular Ca²⁺ influx in vascular endothelial cells, we propose that a similarity between TRPV4 activators and ATP might be crucial for their function. Coincidentally, all 3 TRPV4 activators, including vildagliptin, apigenin, and GSK1016790A, bind to 3 common sites, which has been reported to be critical to ATP binding. Mutation of lysine 340 into alanine significa ntly erased the activation effects of vildagliptin on TRPV4-mediated Ca²⁺ influx, indicating a critical role of the hydrogen bond in the modulation of TRPV4 activity. However, other DPP-4 inhibitors tested, including linagliptin, sitagliptin, saxagliptin, and teneligliptin could not form this hydrogen bond with lysine 340. In addition, because TRPV4 inhibitor HC067047 fails to completely block Ca²⁺ influx induced by vildagliptin, other ion channels may also be involved in this process. Therefore, whether TRPV4 accounts for all of the GLP-1-independent effects of DPP-4 inhibitors still need further determined.

SIRT1 has been reported to display various of benefits on preventing cellular senescence, enhancing vasodilatory responses and attenuating aging-induced vascular damages. In this study, we provide evidence showing that inhibition of SIRT1 by Ex527 abolished the protective role of vildagliptin against hyperglycemia-induced endothelial dysfunction, whereas activation of SIRT1 by SRT1720 improved EDR in absence of TRPV4, demonstrating that SIRT1 acts as a downstream target of TRPV4 in mediating the effect of vildagliptin. Considering that these compounds might induce general reactions in the whole body, it is more ideal to perform these experiments in endothelial-specific SIRT1 transgene or knockout mice. To remedy this limitation, adenovirus-mediated SIRT1 overexpression was applied in in vitro experiments combined treatment of SIRT1 activator, and we not only successfully verified the main conclusion from in vivo studies but also observed a previous unrecognized interaction between SIRT1 and TRPV4. Therefore, our findings imply that although SIRT1 could be stimulated by elevated intracellular Ca²⁺ level, it might also reduce excessive extracellular Ca²⁺ influx in endothelial cells to prevent calcium overload, thus maintain an appropriate intracellular Ca²⁺ level. Nevertheless, further investigation should focus on the exact role of SIRT1 in modulating endothelial Ca²⁺ level.

However, we should also notice that some clinical trials show that DPP-4 inhibitors have less protective effect on cardiovascular function, including saxagliptin, sitagliptin, alogliptin, and linagliptin. One recent study has also shown neutral effect of vildagliptin on endothelial function and arterial stiffness, although its sample number was relatively small and other drugs were included. In addition, the dosage of vildagliptin intervention used in the present study was higher than doses that are used clinically. Therefore, whether or not there exist DPP-4 inhibitors with protective effects on cardiovascular function remains to be confirmed by further clinical trials.

**Perspectives**

This study identified TRPV4 as a direct target of vildagliptin in mediating its protective effect on hyperglycemia-induced endothelial dysfunction. The present study reveals a detailed GLP-1-independent mechanism of vildagliptin in improvement of vascular function and provides a mechanistic insight into the link between endothelial Ca²⁺ homeostasis and diabetic vasculopathy, not only extending the indication of DPP-4 inhibitors to diabetic vascular diseases, but also finding a previously unrecognized interaction between TRPV4 and SIRT1 in the subtle control of endothelial Ca²⁺ homeostasis.

**Acknowledgments**

We thank Anlong Wang and Tingbing Cao for technical assistance. We also greatly appreciated Prof. Xin Ma from School of Pharmaceutical Sciences, Jiangnan University, Wuxi, China for kindly providing us the Transient Receptor Potential Vanilloid 4 knockout mice.

**Sources of Funding**

This work was supported by grants from National Natural Science Foundation of China (81630015 [Z. Zhu], 31871199 [P. Gao], and 81721001 [Z. Zhu]), Innovative Research Team in University (IRT1216; Z. Zhu), and theNovartis DPP-4 Science Award Program (Z. Zhu). L. Li’s work was supported by the grant from CAMS Innovation Fund for Medical Sciences (CIFMS; 2016-I2M-3-007), the China Scholarship Council (201808110107), and the Drug Innovation Major Project (2018ZX09711001-003-005).

**Disclosures**

None.

**References**

1. Versari D, Daghini E, Virdis A, Ghiadoni L, Taddei S. Endothelial dysfunction as a target for prevention of cardiovascular disease. *Diabetes Care*. 2009;32(suppl 2):S314–S321. doi: 10.2337/dc09-S330
2. Nathan DM, Cleary PA, Betlin MA, Neil HA, Matthews DR. Long-term follow-up after tight control of blood pressure in type 2 diabetes. *N Engl J Med.* 2005;353:2643–2653. doi: 10.1056/NEJMoa052187
3. Holman RR, Paul SK, Bethel MA, Neil HA, Matthews DR. Long-term follow-up after tight control of blood pressure in type 2 diabetes. *N Engl J Med.* 2008;359:1565–1576. doi: 10.1056/NEJMoa0806359
4. Allen DA, Yaqoub MM, Harwood SM. Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications. *J Nutr Biochem.* 2005;16:705–713. doi: 10.1016/j.jnutbio.2005.06.007
5. Minamino T, Komuro I. Vascular cell senescence: contribution to atherosclerosis. *Circ Res.* 2007;100:15–26. doi: 10.1161/01.RES.0000256837.40544.4a
null
2 diabetes taking alogliptin versus placebo in EXAMINE: a multicentre, randomised, double-blind trial. *Lancet.* 2015;385:2067–2076. doi: 10.1016/S0140-6736(14)62225-X

42. Rosenstock J, Kahn SE, Johansen OE, Zinman B, Espeland MA, Woerle HJ, Pfarr E, Keller A, Maunder D, et al; Carolina Investigators. Effect of linagliptin vs glimepiride on major adverse cardiovascular outcomes in patients with type 2 diabetes: The Carolina randomized clinical trial [published online September 19, 2019]. *JAMA.* doi: 10.1001/jama.2019.13772 https://jamanetwork.com/journals/jama/fullarticle/10.1001/jama.2019.13772

43. Cosenso-Martin LN, Giollo-Júnior LT, Fernandes LAB, Cesarino CB, Nakazone MA, Machado MN, Yugar-Toledo JC, Vilela-Martin JF. Effect of vildagliptin versus glibenclamide on endothelial function and arterial stiffness in patients with type 2 diabetes and hypertension: a randomized controlled trial. *Acta Diabetol.* 2018;55:1237–1245. doi: 10.1007/s00592-018-1204-1

What Is New?

- Vildagliptin improves endothelial-dependent vasorelaxation in a GLP-1 (glucagonlike peptide-1)-independent manner.
- Transient Receptor Potential Channel Vanilloid 4 (TRPV4) as a direct target of vildagliptin mediates the influx of Ca^{2+} into vascular endothelial cells.
- TRPV4 and SIRT1 (Sirtuin 1) together maintain the Ca^{2+} homeostasis of vascular endothelial cells.

What Is Relevant?

- Our work explains the molecular mechanism of vildagliptin in improving endothelium-mediated vasodilation by activating TRPV4, which provides a theoretical basis for the treatment of vascular dysfunction in diabetes mellitus.

Summary

Vildagliptin improves hyperglycemia-induced endothelial dysfunction by increasing TRPV4-mediated Ca^{2+} uptake, thus activates AMPK (AMP-activated protein kinase)/SIRT1 pathway, which counterbalances the transcription of TRPV4 to maintain an appropriate Ca^{2+} level.

Novelty and Significance