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ORIGINAL ARTICLE

Vascular effects of linagliptin in non-obese diabetic mice are glucose-independent and involve positive modulation of the endothelial nitric oxide synthase (eNOS)/caveolin-1 (CAV-1) pathway

Valentina Vellecco PhD[1†] | Emma Mitidieri PhD[1†] | Antonella Gargiulo PhD student[1†] | Vincenzo Brancaleone PhD[2] | Danilo Matassa PhD[3] | Thomas Klein PhD[4] | Franca Esposito MD[3] | Giuseppe Cirino PhD[1] | Mariarosaria Bucci PhD[1]

[1]Department of Pharmacy, University of Naples "Federico II", Naples, Italy

[2]Department of Science, University of Basilicata, Potenza, Italy

[3]Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", Naples, Italy

[4]Boehringer Ingelheim Pharma GmbH & Co. KG Cardio-metabolic Diseases, Ingelheim am Rhein, Germany

Corresponding Author: Prof. Giuseppe Cirino, Department of Pharmacy, University of Naples "Federico II", via Domenico Montesano, 49 80131 Naples, Italy (cirino@unina.it).

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Aim: To test the effect of linagliptin in non-obese diabetic (NOD) mice, a murine model of type 1 diabetes, to unveil a possible direct cardiovascular action of dipeptidyl peptidase 4 (DPP-4) inhibitors beyond glycaemia control.

Methods: NOD mice were grouped according to glycosuria levels as NODI: none; NODII: high; NODIII: severe. Linagliptin treatment was initiated once they reached NODII levels. Vascular reactivity was assessed *ex vivo* on aorta harvested from mice upon reaching NODIII level. In a separate set of experiments, the effect of linagliptin was tested directly *in vitro* on vessels harvested from untreated NODIII, glucagon-like peptide-1 (GLP-1) receptor knockout and soluble guanylyl cyclase-α1 knockout mice. Molecular and cellular studies were performed on endothelial and endothelial nitric oxide synthase (eNOS)-transfected cells.

Results: In this *ex vivo* vascular study, endothelium-dependent vasorelaxation was ameliorated and eNOS/nitric oxide (NO)/soluble guanylyl cyclase (sGC) signalling was enhanced. In the *in vitro* vascular study, linagliptin exerted a direct vasodilating activity on vessels harvested from both normo- or hyperglycaemic mice. The effect was independent from GLP-1/GLP-1 receptor (GLP-1R) interaction and required eNOS/NO/sGC pathway activation. Molecular studies performed on endothelial cells show that linagliptin rescues eNOS from caveolin-1 (CAV-1)-binding in a calcium-independent manner.

Conclusion: Linagliptin, by interfering with the protein–protein interaction CAV-1/eNOS, led to an increased eNOS availability, thus enhancing NO production. This mechanism accounts for the vascular effect of linagliptin that is independent from glucose control and GLP-1/GLP-1R interaction.

KEYWORDS

caveolin-1, DPP-4 inhibitors, GLP-1 receptor, NOD mice, soluble guanylyl cyclase

1 | INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by enteroendocrine cells in response to meals in order to reduce postprandial hyperglycaemia.[1,2] Dipeptidyl peptidase-4 (DPP-4) rapidly inactivates incretins with a half-life of a few minutes[3]; therefore, DPP-4 inhibitors or gliptin are currently used in the treatment of type 2 diabetes mellitus (T2DM). Recently, both experimental and clinical studies have suggested that DPP-4 inhibitors can modulate cardiovascular function[4–7] by ameliorating ischaemia-reperfusion injury,[8,9] reducing

[†]These authors contributed equally to the study.

the atherosclerotic lesions[10] and vascular inflammatory reaction,[11,12] and protecting the heart from acute myocardial ischaemia.[7,13] There are a number of underlying molecular mechanisms involved in angiopathy associated with diabetes that are recognized, where there is impaired nitric oxide (NO) bioavailability, to be the main cause of endothelial dysfunction. It has been shown, in both preclinical[14–17] and clinical studies,[18–21] that DPP-4 inhibitors positively modulate endothelium function, but the molecular mechanism through which DPP-4 inhibitors exert this effect is still unknown. To date, both a GLP-1 receptor (GLP-1R)-dependent effect and the independent effect of gliptins have been suggested. The GLP-1R-dependent hypothesis is based on the finding that DPP-4 inhibition, by inducing an increase in GLP-1, promotes its interaction with GLP-1R expressed in the endothelium.[22,23] This event activates a downstream signal transduction that includes cAMP production and K_{ATP} channel activation.[24,25] The receptor-independent hypothesis postulates a direct involvement of DPP-4 in vascular reactivity. In line with this latter hypothesis, it has been shown that DPP-4 is expressed in the vascular endothelium[25] and its inhibition induces a direct vasorelaxant effect on isolated vessels *in vitro* in the presence of GLP-1R antagonist.[14,15]

By overviewing the literature regarding the protective cardiovascular effect of DPP-4 inhibitors, it appears evident that all the studies have been performed in T2DM. This consideration highlights the difficulty in separating the beneficial effect of DDP-4 inhibitors on cardiovascular function aside from their action on glycaemia. In other words, it is difficult to exclude the hypothesis that the cardiovascular effect of DPP-4 inhibitors is not secondary to their hypoglycaemic action. To clarify this issue, we performed an experimental study in which DPP-4 inhibitors were tested in a murine model of type 1 diabetes mellitus (T1DM), i.e. non-obese diabetic (NOD) mice. Because of the lack of effect of this class of drugs on T1DM, this animal model allowed us to dissect the DPP-4 inhibitors' direct cardiovascular action from the indirect beneficial effect attributable to the reduction in hyperglycaemia.

2 | MATERIALS AND METHODS

2.1 | Animals

All animal procedures were performed according to the Declaration of Helsinki (European Union guidelines on use of animals in scientific experiments) and the ARRIVE guidelines, and the study was authorized by the local animal care office (Centro Servizi Veterinari, Università degli Studi di Napoli "Federico II"). This work was carried out on NOD/Ltj and age-matched CD-1 control mice (Charles River, Calco, Italy). GLP-$1R^{-/-}$ and soluble guanylyl cyclase-α1 knockout ($sGC_{\alpha1}{}^{-/-}$) mice were also used and supplied by Boheringer Ingelheim (Ingelheim, Germany) and Prof. P. Brouckaert from the University of Ghent (Belgium), respectively. Mice were kept in an animal care facility under controlled temperature, humidity and light/dark cycle conditions, with food and water *ad libitum*.

2.2 | NOD/Ltj mice

NOD/Ltj mice spontaneously develop T1DM,[26] which was assessed through measurement of glycosuria (monitored weekly) by using glucose-6-oxidase-based assay (Biogamma, Roma, Italy). Mice were divided into three groups based on glycosuria level (Figure 1A,B): NODI: glycosuria <20 mg/dL = low/none; NODII: glycosuria 20-500 mg/dL = high; NODIII: glycosuria 500-1000 mg/dL = severe.

Oral treatment with linagliptin (0.3 and 1 mg/kg, daily) was started when mice reached NODII stage. The mice were killed when NODIII glycosuria level was achieved.

2.3 | Isolated organ bath studies

2.3.1 | *Ex vivo*

Once at NODIII level, mice treated with linagliptin as described above, were sacrificed by exposure to CO_2 and the aorta was rapidly

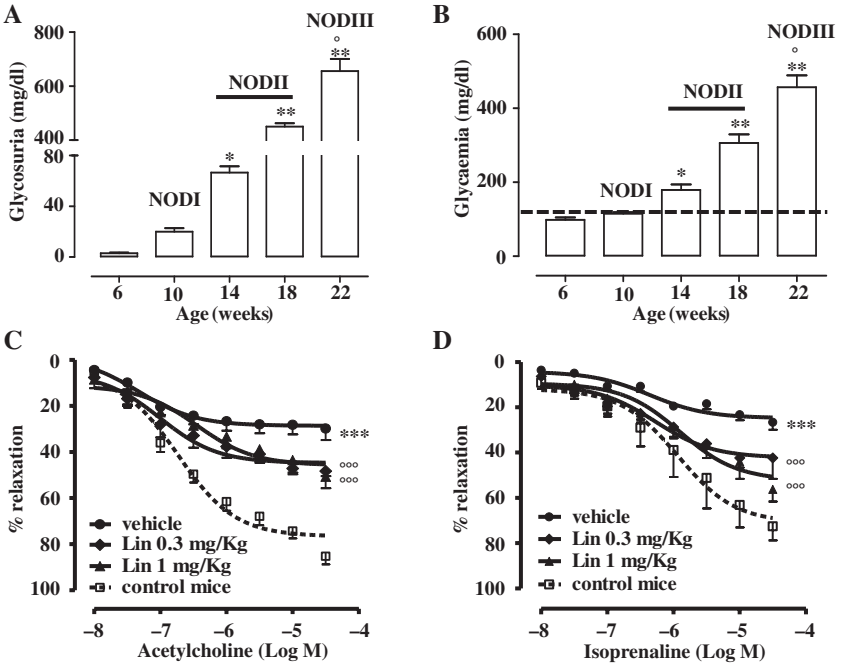

FIGURE 1 A, Classification of NOD mice based on glycosuria and B, glycaemia value (n = 6-14; *P < .05, **P < .01 vs 6 week; °P < .05 vs NODII). NODII mice (e.g. when glycaemia is already at pathological concentration) were treated with linagliptin (0.3 and 1 mg/kg) until they reached NODIII stage. During the treatments, glycosuria was monitored twice a week and mice were killed when they reached NODIII stage. C, Effect of linagliptin treatment (0.3, 1 mg/kg) on acethylcholine-induced vasorelaxation in aortic rings (n = 6 mice). D, Effect of linagliptin treatment (0.3, 1 mg/kg) on isoprenaline-induced vasorelaxation in aortic rings (n = 6 mice). Data are presented as mean ± s.e.m. ***P < .001 vs age-matched control mice (normoglycaemic), and °°°P < .001 vs linagliptin vehicle (natrosol).

dissected and cleaned from connective tissue. Aorta rings were cut, mounted in an isolated organ bath filled with oxygenated Krebs solution at 37°C, and connected to isometric transducers. Changes in tension were recorded continuously with a computerized system (DataCapsule 17400; Ugo Basile, Comerio, Italy). Rings were initially stretched until a resting tension of 1.5 g was reached and allowed to equilibrate for 45 minutes. Concentration–response curves to acethylcholine (acethylcholine) and isoprenaline (10 nM to 30 μM) were performed on phenylephrine pre-contracted rings (1 μM).

2.3.2 | *In vitro*

Mice were killed by exposure to CO_2 and aorta were rapidly harvested. We assessed concentration–response curves of linagliptin and sitagliptin (100 nM to 30 μM) in the presence or absence of endothelium and in the presence of N^5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO 10 μM, 15 minutes), 1*H*-[1,2,4]Oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ 10 μM; 15 minutes) or vehicle.

2.4 | Cell culture experiments in a normal and high-glucose environment

Human embryonic kidney cells stably transfected with eNOS (HEK-293st) and bovine aortic endothelial cells (BAEC) were cultured and grown in medium supplemented with 2 mmol/L glutamine, 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin/streptomycin. HEK-293st were incubated with linagliptin (0.1-100 nM) or vehicle [phosphate-buffered saline (PBS)] for 1 hour and then incubated for 2 hours in 25 mM (normal glucose) or 50 mM (high glucose) D-glucose solution.[27] Cells were then stimulated with calcium ionophore A23187 (10 μM, 30 minutes). Cell pellets were collected and used for Western blot analysis, while supernatants were used for nitrite/nitrate (NOx) assay. The same protocol was applied in experiments with BAEC.

2.5 | Immunoprecipitation study

Immunoprecipitation analysis was performed on BAEC (300 μg total protein). Lysates were pre-cleared by incubating with protein A/G-Agarose (SantaCruz Biotechnology, Hidelberg, Germany) for 1 hour at 4°C and then incubated under agitation for 18 hours at 4°C with the relevant antibodies. Subsequently, samples were further incubated for 1 hour at 4°C with fresh beads, washed in PBS and collected by centrifugation. Mouse monoclonal anti-eNOS (1:500, BD-Trasduction Laboratories, Milano, Italy) and rabbit polyclonal anti-caveolin-1 (CAV-1, 1:1000, Santa Cruz Biotechnology) were used as primary antibodies.

2.6 | Western blotting

Aorta or HEK-293st were homogenized in RIPA buffer. Denatured proteins (40 μg) were separated on 10% sodium docecyl sulphate-polyacrylamide gel. Membranes were blocked in PBS containing 0.1% v/v Tween-20 and 3% w/v non-fat dry milk for 30 minutes, followed by overnight incubation at 4°C with rabbit polyclonal anti-CAV-1 (1:1000) or mouse monoclonal anti-eNOS (1:500). Membranes were washed in PBST prior to incubation with horseradish-peroxidase-conjugated secondary antibody for 2 hours. After incubation, membranes were washed and developed using a chemiluminescence assay. Images were obtained busing ImageQuant-400 (GE Healthcare, Chicago, IL, USA). The housekeeping protein GAPDH (1:5000, Sigma-Aldrich, Milan, Italy) was used for normalization.

2.7 | NOx assay

Assessment of NOx levels was performed according to Misko et al.[28] with modifications. Briefly, cellular supernatants (120 μL) were incubated with cadmium (50 mg/well) in a microplate for 1 hour to convert NO_3^- into NO_2^-. After centrifugation, total NOx content was determined using a fluorometric method[29] and calculated against a standard curve of sodium nitrite (50-2000 nM).

2.8 | Measurement of intracellular Ca^{+2}

Changes in the intracellular Ca^{+2} concentration were measured in BAEC using a Fluo-4 NW Calcium Assay Kit (Molecular Probes, Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Fluorescence (excitation 485 nm, emission 525 nm) was measured for 3 minutes in a microplate fluorometer (Promega, Madison, WI, USA).

2.9 | Statistical analysis

All data were expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post-test, or two-way ANOVA followed by Bonferroni's post-test (GraphPad software, San Diego, CA, USA). Differences with p values <.05 were taken to indicate statistical significance.

3 | RESULTS

3.1 | Linagliptin treatment ameliorates endothelium-dependent vasorelaxation in NOD mice

We have previously shown that in NOD mice, the development of T1DM is coupled with a progressive impairment of endothelium-dependent vasorelaxation.[27] Indeed, acethylcholine-induced vasorelaxation is strongly blunted in NODIII mice, with a shift of the E_{max} (maximal effect) from 85.3 ± 3.6% in control mice to 29.8 ± 5.0% in NODIII. Similarly, isoprenaline-induced vasorelaxation results in strongly impaired NODIII mice with a shift of the E_{max} from 72.5 ± 6.3% in control mice to 26.6 ± 3.3% in NODIII. In aorta rings harvested after linagliptin treatment (0.3 and 1 mg/kg), both acethylcholine- (Figure 1C) and isoprenaline- (Figure 1D) induced vasorelaxation are improved, with a significant increase in E_{max} (50.7 ± 5.03% and 56.3 ± 5.4% for acethylcholine and isoprenaline, respectively).

3.2 | Linagliptin treatment enhances nitric oxide signalling

Previously, we have shown that an increased expression of CAV-1 occurs in diabetes, contributing to reduced endothelium-dependent vasodilatation.[27] We therefore evaluated the effect of linagliptin treatment on CAV-1 and eNOS expression. As shown in Figure 2A,B, linagliptin modulates the expression of these two proteins towards a more active state. Indeed, in aorta homogenates, eNOS analysis shows a trend of enhanced expression after linagliptin treatment (Figure 2A), as opposed to CAV-1, which shows a trend of reduced expression (Figure 2B). These data, taken together, suggest that linagliptin exerts a positive action on eNOS/NO signalling.

3.3 | eNOS/CAV-1 involvement in the linagliptin effect in HEK-293 stably transfected with eNOS (HEK-293st)

We have previously shown that a high-glucose environment blunts eNOS activity[27] (e.g. NO production) in HEK-293st (Figure 2C). This effect correlates with increased CAV-1 expression, coupled with a reduction in eNOS content (Figure 2D,E). Linagliptin incubation of HEK-293st in a high-glucose environment (0.1-100 nM) completely restores the physiological pattern observed in normoglycaemia. Indeed, NO release showed a recovery trend, reaching statistical significance at the maximum concentration of 100 nM of linagliptin (Figure 2C). This effect is accompanied by a significant reduction in CAV-1 expression (Figure 2D,E). Linagliptin (0.1-100 nM) exerts a similar effect when tested on BAEC. Indeed, in BAEC placed in a high-glucose environment, linagliptin restores nitrite levels observed in normoglycaemia (Figure 5A).

3.4 | Linagliptin has a direct vasodilating activity and acts through eNOS/cGMP signalling

As shown in Figure 3A, linagliptin can directly induce vasodilatation *in vitro* in a concentration-dependent manner (100 nM to 30 μM) on aorta harvested from control mice. Sitagliptin induces vasodilatation as well, but is less active (E_{max} 39.4 ± 7.3 % vs 89.4 ± 2.7%, sitagliptin vs linagliptin, respectively; Figure S1). As shown in Figure 3A, in the absence of endothelium, linagliptin-induced vasodilatation is significantly impaired. This result suggests that the endothelium is the main player in linagliptin-induced vasodilatation. Incubation of aorta rings with L-NIO, a selective eNOS inhibitor, significantly reduces linagliptin-induced vasorelaxation (Figure 3B). Similarly, incubation with ODQ, an inhibitor of NO-dependent sGC activity, significantly inhibits linagliptin-induced vasorelaxation (Figure 3B).

The same panel of *in vitro* experiments have been performed on aorta harvested from mice with severe glycosuria, i.e. NODIII mice. As shown in Figure 3C, linagliptin induces a concentration-dependent vasodilatation in aortic rings of NODIII mice, although to a minor extent compared with control mice. Similar to that which was observed in control mice, when aortic rings are incubated with L-NIO or ODQ, linagliptin-induced vasodilatation results significantly reduced (Figure 3D), confirming the involvement of NO release in linagliptin vasodilating action.

3.5 | Linagliptin-induced vasodilatation is independent from GLP-1 receptor

To clarify the possible involvement of GLP-1R in linagliptin-induced vasorelaxation, the same experimental protocol used for the *in vitro* study was performed on aorta harvested from GLP-1R$^{-/-}$ mice and

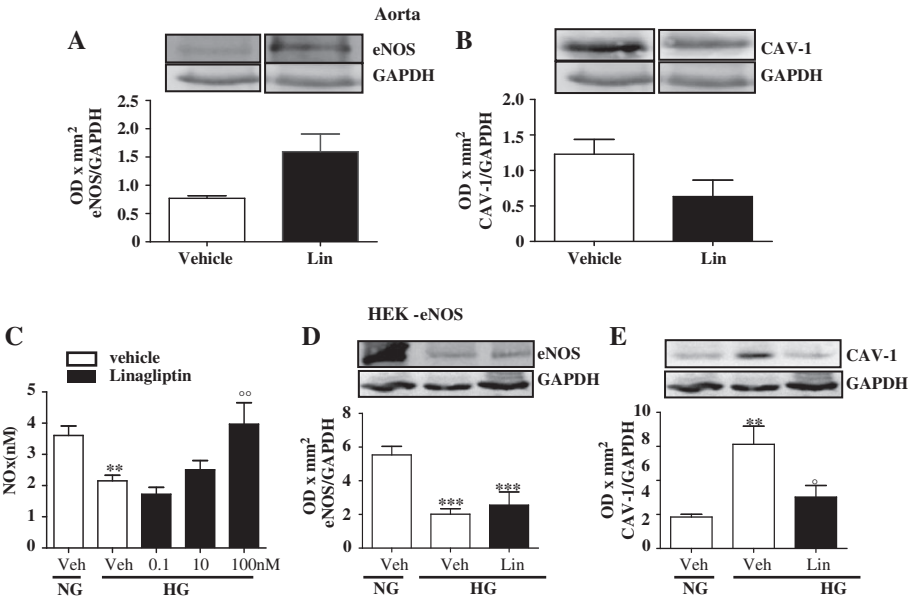

FIGURE 2 A,B, Representative western blot images of eNOS and CAV-1 performed on aorta homogenates harvested from NODIII mice after treatment with linagliptin (Lin; 1 mg/kg) *in vivo*. Data are compared with NODIII mice treated with vehicle (n = 3 for each group). C, Effect of linagliptin (0.1, 10, 100 nM) on Ca^{2+} ionophore A23187-induced NOx levels in HEK-293st in hyperglycaemic condition (HG; n = 3 for each group). HEK-293st treated with vehicle in hyperglycaemic and normoglicaemic (NG) conditions were used as controls. $^{**}P < .01$ vs vehicle in the normoglycaemic condition and $^{\circ\circ}P < .01$ vs vehicle in the hyperglycaemic condition. D,E, Representative Western blot images of eNOS and CAV-1 in HEK-293st in the hyperglycaemic condition after treatment with vehicle or linagliptin (100 nM); data are compared with HEK-293st in the normoglycaemic condition treated with vehicle (n = 3 for each group). $^{**}P < .01$ and $^{***}P < .001$ vs vehicle in the normoglycaemic condition and $^{\circ}P < .05$ vs vehicle in the hyperglycaemic condition.

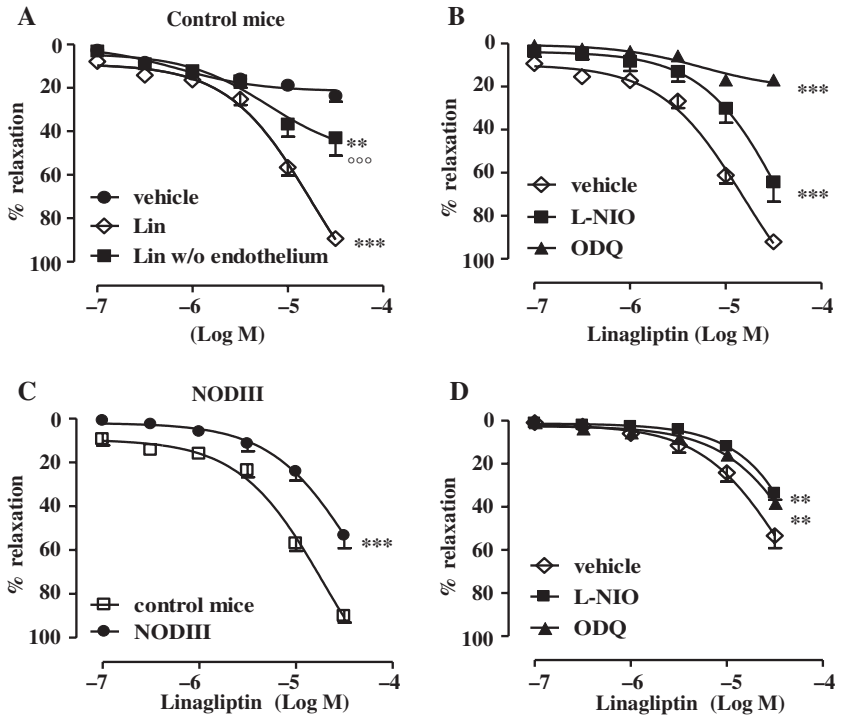

FIGURE 3 A, Concentration–response curve of linagliptin (0.1-30 μM) on aortic rings with or without endothelium from control mice (n = 6 mice for each group). $^{**}P < .01$ and $^{***}P < .001$ vs vehicle, $^{\circ\circ\circ}P < .001$ vs linagliptin. B, Effect of L-NIO (10 μM) and of ODQ (10 μM) on linagliptin-induced vasorelaxation on control mice (n = 6 mice for each treatment). $^{***}P < .001$ vs vehicle. C, Concentration–response curve of linagliptin (0.1-30 μM) on aortic rings harvested from NODIII mice. Control mice were used as normoglycaemic control (n = 6 mice). $^{***}P < .001$ vs control mice. D, Effect of L-NIO (10 μM) and of ODQ (10 μM), on linagliptin-induced vasorelaxation on NODIII mice (n = 6 mice for each treatment). $^{**}P < .01$ vs vehicle. Data are presented as mean ± s.e.m.

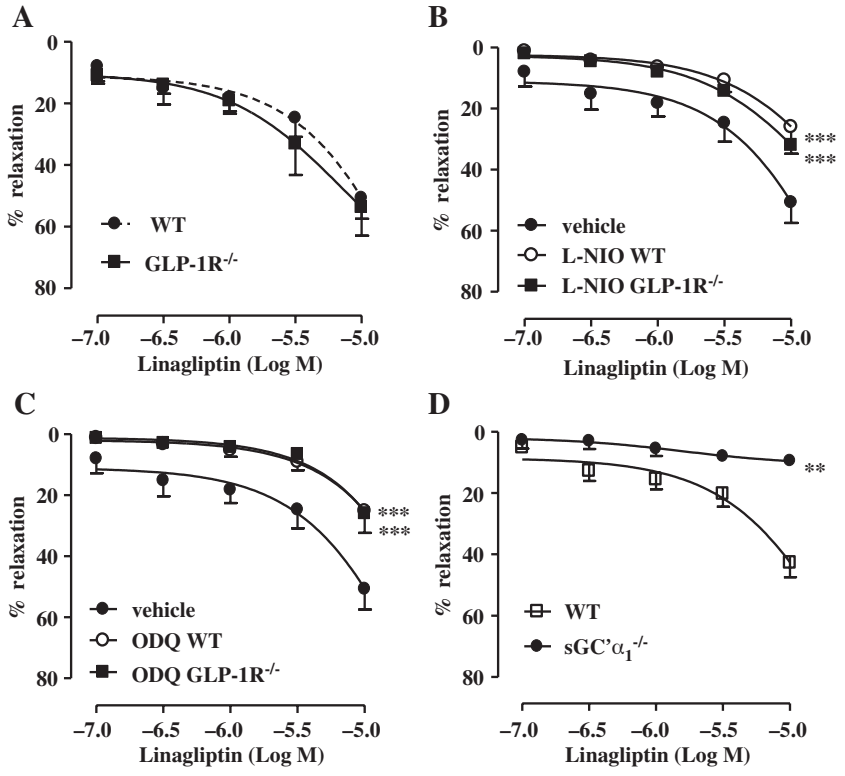

FIGURE 4 A, Concentration–response curve of linagliptin (0.1-10 μM) on aortic rings harvested from GLP-1R$^{-/-}$ mice and respective wild-type (WT) mice. B, Effect of L-NIO (10 μM) on linagliptin-induced vasodilation in GLP-1R$^{-/-}$ and WT mice. C, Effect of ODQ (10 μM) on linagliptin-induced vasodilation in GLP-1R$^{-/-}$ and WT mice. D, Concentration–response curve of linagliptin (0.1-10 μM) on aortic rings harvested from sGCα1$^{-/-}$ mice compared with WT mice (n = 6 mice for each group). $^{***}P < .001$ vs vehicle, $^{\circ\circ}P < .01$ vs WT. Data are presented as mean ± s.e.m.

on their matched wild-type controls. Our results show that linagliptin-induced vasodilatation is completely preserved in aortic rings of GLP-1R$^{-/-}$ (Figure 4A). This evidence rules out GLP-1R involvement from linagliptin-induced vasodilatation, reinforcing our hypothesis of direct action of linagliptin on vascular function, independent from GLP-1/GLP-1R interaction. L-NIO and ODQ significantly reduce linagliptin-induced vasodilatation also in GLP-1R$^{-/-}$ mice (Figure 4B,C).

3.6 | Linagliptin fails to induce vasodilatation in soluble guanylyl cyclase α1 knockout mice

To further validate the key role of NO in linagliptin-induced vasodilatation, we assessed the cumulative concentration–response curve on aorta harvested from sGC$_{\alpha 1}{}^{-/-}$ mice.[30,31] As shown in Figure 4D, linagliptin-induced vasodilatation is significantly impaired, confirming the involvement of the NO pathway and its downstream signalling in linagliptin vasodilating action.

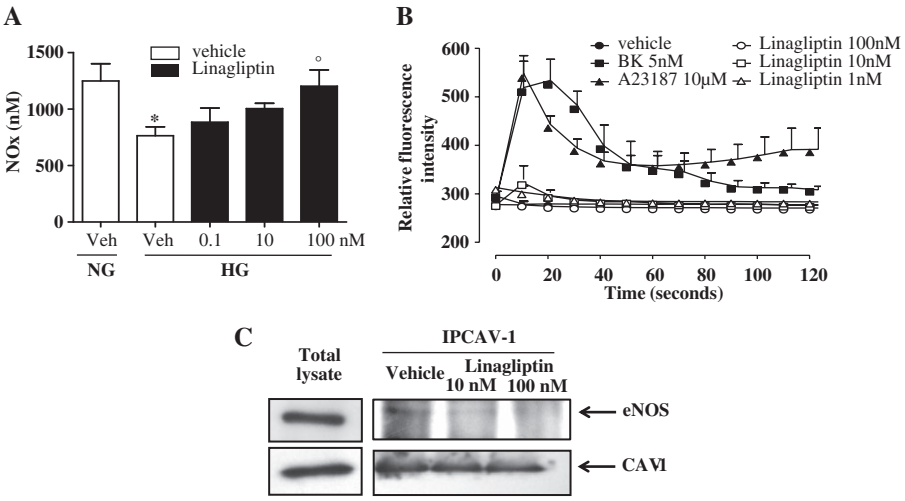

FIGURE 5 A, Effect of linagliptin (0.1, 10, 100 nM) on Ca^{+2} ionophore A23187-induced NOx levels in BAEC placed in a hyperglycaemic condition. Data are compared with BAEC treated with vehicle in hyperglycaemic and normoglycaemic conditions. NG (normal glucose) HG (high glucose; n = 3). B, Effect of linagliptin (1, 10, 100 nM) on intracellular Ca^{+2} influx in BAEC (n = 3). Data are compared with BAEC stimulated with vehicle and expressed as relative fluorescence. BK and A23187 were used as positive control. C, Immunoprecipitation (IP) of CAV-1 and western blot of eNOS in BAEC treated with linagliptin (10 and 100 nM); as can be seen, immunoprecipitation of CAV-1 shows that treatment with linagliptin removes eNOS from CAV-1. Compare line 1 with lines 2 and 3. Data are compared to untreated BAEC (n = 3).

3.7 | Linagliptin does not modify intracellular Ca^{+2} influx but interferes with the eNOS/CAV-1 complex in BAEC

To assess the role of $[Ca^{+2}]_i$ in the linagliptin molecular effect, we measured $[Ca^{+2}]_i$ levels after linagliptin stimulation. When BAEC were treated with linagliptin, no significant rise in $[Ca^{+2}]_i$ was observed (Figure 5B). Bradykinin and A23187 were used as positive controls. As shown in Figure 5C, in untreated cells, a protein band immunoreactive to anti-eNOS antibody can be easily detected in the CAV-1 immunoprecipitation. Treatment with linagliptin (10 and 100 nM) significantly reduces the eNOS co-immunoprecipitation. The effect is concentration-dependent; e.g. the highest concentration of linagliptin induced the lowest interaction between eNOS and CAV-1.

4 | DISCUSSION

The concept that DPP-4 inhibitors could ameliorate vascular reactivity in diabetes has already been assessed in both clinical and experimental studies.[4–7,14–17] The selection of a T1DM animal model (NOD mice) to investigate the vascular effect of DPP-4 inhibitors was based on the finding that NOD mice: (1) spontaneously develop an autoimmune diabetes with remarkable analogy to human T1DM[26,27]; and (2) develop a progressive impaired vascular reactivity, which we have previously characterized.[27] Taking advantage of the fact that DPP-4 inhibitors do not control glycaemia in T1DM, this strain represents an appropriate tool with which to investigate the effect of DPP-4 inhibitors on vascular complications, minimizing the confounding variable attributable to the beneficial effect that a reduction of glycaemia would have by itself on vessels.

The impairment of NO-dependent vasodilation represents one of the key events in the diabetes-induced vascular dysfunction in these mice. Indeed, endothelium-dependent relaxation elicited by either acethylcholine or isoprenaline are severely impaired in NODIII mice and their endothelial-dependent effect is mediated by eNOS/NO/sGC pathway activation.[32–34] Treatment *in vivo* with linagliptin significantly improves the NO-dependent vasodilatation *ex vivo*, despite the lack of control of glycaemia. In fact, as expected, the progressive, age-related increase in glycosuria observed in NOD mice, was not significantly affected by linagliptin treatment. This finding raises the possibility that DPP-4 inhibitors could have a direct effect on vessels. It has been previously shown that DPP-4 inhibitors induce vasodilatation on isolated rat[15] and mouse aorta.[14] In particular, Kroller-Schon et al.[15] have shown, that different DPP-4 inhibitors can directly dilate the aorta *in vitro*, suggesting this "direct" effect is a class effect. To define this issue, we switched from *ex vivo* to *in vitro* experiments. Our *in vitro* experiments were designed to define the molecular mechanism of DPP-4 inhibitor-induced vasodilatation and to compare the DPP-4 inhibitor-induced vasodilatation in healthy and diabetic conditions. The study was performed using sitagliptin, belonging to the dipeptide mimetic structure subclass, and linagliptin, belonging to the non-peptidomimetic DPP-4 sub-class. Linagliptin has a concentration-dependent relaxation effect on healthy vessels, with a E_{max} of almost 90%. Sitagliptin also has a relaxant effect but with an E_{max} that is less than half that of linagliptin (Figure S1). The pharmacological modulation studies performed *in vitro* using linagliptin only, because it induces the best vasodilatation, clearly showed that the linagliptin effect involves eNOS/NO/sGC. Indeed, inhibition of either eNOS or sGC leads to a significant reduction of linagliptin-induced vasorelaxation. One important issue is that the relaxant effect of linagliptin is retained in the presence of dysfunctional endothelium, e.g. in aorta harvested from NODIII mice. These data suggest that the mechanism underlying the DPP-4 inhibitor effect is directed to the eNOS/NO/sGC pathway. The involvement of the eNOS/NO pathway in the linagliptin effect is further confirmed by the finding that linagliptin-induced vasodilatation *in vitro* is strongly impaired in the aorta from $sGC\alpha_1^{-/-}$ mice. It is known that $sGC\alpha_1\beta_1$ heterodimer is the most abundant sGC isoform in the cardiovasculature,[30] and mice

lacking $sGC\alpha_1^{-/-}$ develop hypertension.[31] These data, taken together with the finding that DPP-4 inhibitor-induced vasorelaxation is completely preserved in $GLP\text{-}1R^{-/-}$ mice, show that the direct vasodilating effect of linagliptin is eNOS/NO/sGC-dependent and unrelated to GLP-1/GLP-1R. The independence of the linagliptin vasoactive effect from GLP-1R is also corroborated by other studies where its beneficial effect in stroke is also disjointed from GLP-1R activation.[35] The hypothesis that DPP-4 inhibitors promote eNOS activation is already present in the literature[16,17]; however, the molecular mechanism is as yet not clear. Previously, we have shown that eNOS impairment in NODIII mice vessels is attributable to an increased expression of CAV-1, a resident caveolae protein that negatively regulates eNOS.[27] Because both the *ex vivo* and the *in vitro* data pointed to an effect on eNOS, we investigated the possibility that linagliptin could interact with eNOS-CAV-1 complex. To this end, in a separate set of experiments, aortas were harvested after *in vivo* linagliptin treatment and molecular analysis was performed. The Western blot analysis showed that eNOS tended to be upregulated, while CAV-1 had the opposite profile, suggesting that more eNOS is in its active state. To address this issue in depth, we used HEK-293 stably transfected with eNOS. $HEK\text{-}293^{st}$ cells cultured in high glucose levels, show an increase in CAV-1 expression coupled with reduced eNOS expression. This pattern of expression correlates well with a reduction in NO levels.[27] Linagliptin treatment significantly inhibits CAV-1 expression, almost restoring it to normoglycaemic levels. All these data point to CAV-1/eNOS complex disruption as the molecular event responsible for the beneficial vascular action of linagliptin. Our next step, therefore, was to understand how linagliptin interferes with the CAV-1/eNOS complex. Two possibilities were taken in consideration. First, linagliptin interacts with the endothelial cellular structure (i.e. membrane receptor or channel), acting as a calcium-mobilizing agonist. This intracellular Ca^{2+} elevation augments calmodulin affinity to eNOS, leading to its activation.[36,37] Second, linagliptin directly interacts with the CAV-1/eNOS complex. The Fluo-4 assay performed on BAEC clearly shows that linagliptin is not a calcium-mobilizing agonist because no significant rise in intracellular calcium was observed after linagliptin stimulation. The CAV-1/eNOS immunoprecipitation study shows that linagliptin reduces the CAV-1/eNOS interaction and this effect was concentration-dependent. Of particular relevance is the finding that the concentrations of linagliptin used to achieve this effect *in vitro*, e.g. 10 nM, are within the range of linagliptin circulating plasma levels in humans, making our data clinically relevant.[38]

In conclusion, we show that the DPP-4 inhibitor linagliptin improves vascular function in diabetes independently of glucose control, consistent with a recent paper showing a similar effect on cerebrovascular circulation.[39] This effect does not involve GLP-1 but relies on direct interaction of linagliptin with the eNOS/CAV-1 complex, leading to a rescue of eNOS activity from the negative regulation operated by CAV-1. This molecular event, in turn, causes an increase in NO production with a significant beneficial effect on vascular function. One possible clinical implication is that the therapeutic use of linagliptin could also be associated with a protective effect on the vascular dysfunction associated with endothelial impairment in patients with T2DM; however, this possibility, even though it has been already put forward in the current literature, needs an appropriate clinical study.

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Conflict of interest

T. K. is an employee of Boehringer Ingelheim. The study was also performed with the contribution of Boehringer Ingelheim. V. V., E. M., A. G., D. M., V. B., F. E., M. B., G. C. declare no conflict of interest. G. C. takes responsibility, as guarantor, for the contents of this article.

Author contributions

V. V., E. M. and A. G. performed the experiments and data interpretation. D. M. performed the immunoprecipitation experiments and analysed the data. V. B. performed the *in vitro* experiments on NODIII mice. M. B. conceived and coordinated the experiments. F. E. revised the manuscript and wrote the experimental section on molecular biology. T. K. revised the manuscript and planned the project. G. C. planned and coordinated the project. M.B. and G.C. wrote the manuscript.

REFERENCES

1. Drucker DJ. The biology of incretin hormones. *Cell Metab*. 2006;3:153–165.
2. Campbell JF, Drucker DJ. Pharmacology, physiology, and mechanisms of incretins hormone action. *Cell Metab*. 2013;17:819–837.
3. Mulvihill EE, Drucker DJ. Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. *Endocr Rev*. 2014;35:992–1019.
4. Wang XM, Yang YJ, Wu YJ. The emerging role of dipeptidyl peptidase-4 inhibitors in cardiovascular protection: current position and perspectives. *Cardiovasc Drugs Ther*. 2013;27:297–307.
5. Murohara T. Dipeptidyl peptidase-4 inhibitor: another player for cardiovascular protection. *J Am Coll Cardiol*. 2012;59:277–279.
6. Chrysant SG, Chrysant GS. Clinical implication of cardiovascular preventive pleiotropic effects of dipeptidyl peptidase-4 inhibitors. *Am J Cardiol*. 2012;109:1681–1685.
7. Shigeta T, Aoyama M, Bando YK, et al. Dipeptidyl peptidase-4 modulates left ventricular dysfunction in chronic heart failure via angiogenesis-dependent and –independent actions. *Circulation*. 2012;126:1838–1851.
8. Ku CH, Chen WP, Su MJ. DPP4 deficiency preserve cardiac function via GLP-1 signaling in rats subjected to myocardial ischemia/reperfusion. *Naunyn Schmiedebergs Arch Pharmacol*. 2011;384:197–207.
9. Sauvé M, Ban K, Momen MA, et al. Genetic deletion or pharmacological inhibition of dipeptidyl peptidase 4 improves cardiovascular outcome after myocardial infarction in mice. *Diabetes*. 2010;59:1063–1073.
10. Matsubara J, Sugiyama S, Sugamura K, et al. A dipeptidyl peptidase-4 inhibitor, des-fluoro-Sitagliptin, improbe endothelial function and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. *J Am Coll Cardiol*. 2012;59:265–276.

11. Ervinna N, Mita T, Yasunari E, et al. Anagliptin, a DPP-4 inhibitor, suppresses proliferation of vascular smooth muscle and monocyte inflammatory reaction and attenuates atherosclerosis in male apo E-deficient mice. *Endocrinology*. 2013;154:1260–1270.
12. Terasaki M, Nagashima M, Nohtomi K, et al. Preventive effect of dipeptidyl peptidase-4 inhibitoron atherosclerosis is main attributable to incretin's actions in non diabetic and diabetic apo-E null mice. *PLoS One*. 2013;8:1–12.
13. Gomez N, Matheeussen V, Damoiseaux C, et al. Effect of heart failure on dipeptidyl peptidase IV activity in plasma of dogs. *J Vet Intern Med*. 2012;26:929–934.
14. Shah Z, Pineda C, Kampfrath T, et al. Acute DPP-4 inhibition modulates vascular tone through GLP-1 independent pathways. *Vasc Pharmacol*. 2011;55:2–9.
15. Kroller-Schon S, Knorr M, Hausding M, et al. Glucose-independent improvement of vascular dysfunction in experimental sepsis by dipeptidyl peptidase-4 inhibition. *Cardiovasc Res*. 2012;96:140–149.
16. Huang CY, Shih CM, Tsao NW, et al. Dipeptidyl peptidase-4 inhibitor improves neovascularization by increasing circulating endothelial progenitor cells. *Br J Pharmacol*. 2012;167:1506–1519.
17. Liu L, Liu J, Wong WT, et al. Dipeptidyl peptidase 4 inhibitor Sitagliptin protects endothelial function in hypertension through a glucagon-like peptide 1-dependent mechanism. *Hypertension*. 2012;60:833–841.
18. Stuhlinger MC, Abbasi F, Chu JW, et al. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. *JAMA*. 2002;287:1420–1426.
19. Abbasi F, Asagmi T, Cooke JP, et al. Plasma concentration of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. *Am J Cardiol*. 2001;88:1201–1203.
20. Fadini GP, Boscaro E, Albiero M, et al. The oral dipeptdyl peptidase-4 inhibitor Sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes. *Diabetes Care*. 2010;33:1607–1609.
21. Ogawa S, Ishiki M, Nako K, et al. Sitagliptin, a dipeptidyl peptidase-4 inhibitor, decreases systolic blood pressure in Japanese hypertensive patients with type 2 diabetes. *Tohoku J Exp Med*. 2011;223:133–135.
22. Ban K, Noyan-Ashraf MH, Hoefer J, Bolz SS, Drucker DJ, Husain M. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 -receptor-dependent and –independent pathways. *Circulation*. 2008;117:2340–2350.
23. Ishibasshi Y, Matsui T, Takeuchi M, Yamagishi S. Glucagon-like peptide 1 (GLP-1) inhibits advanced glycation and product (AGE)-induced up-regulation of VCAM-1 mRNA levels in endothelial cells by suppressing AGE receptor (RAGE) expression. *Biochem Biophys Res Commun*. 2010;391:1405–1408.
24. Green BD, Hand KV, Dougan JE, McDonnell BM, Cassidy RS, Grieve DJ. GLP-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving K_{ATP} and cAMP. *Arch Biochem Biophys*. 2008;478:136–142.
25. Pala L, Rotella CM. The role of DPP4 activity in cardiovascular districts: in vivo and in vitro evidence. *J Diabetes Res*. 2013;2013:590456.
26. Makino S, Kunimoto K, Muraoka Y, Mizushima K, Katagiri K, Tochino Y. Breeding of a non-obese diabetic strain of mice. *Jikken Dobutsu*. 1980;29:1–13.
27. Bucci M, Roviezzo F, Brancaleone V, et al. Diabetic mouse angiopathy is linked to progressive sympathetic receptor deletion coupled to an enhanced caveolin-1 expression. *Arterioscler Thromb Vasc Biol*. 2004;24:721–726.
28. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorimetric assay for the measurement of nitrite in biological samples. *Anal Biochem*. 1993;214:11–16.
29. Bucci M, Roviezzo F, Brancaleone V, et al. ACE-inhibition ameliorates vascular reactivity and delays diabetes outcome in NOD mice. *Vascul Pharmacol*. 2008;49:84–90.
30. Mergia E, Russwurm M, Zoidl G, Koesling D. Major occurrence of the new alpha(2)beta(1) isoform of NO-sensitive guanylyl cyclase in brain. *Cell Signal*. 2003;15:189–195.
31. Buys ES, Sips P, Vermeersch P, et al. Gender-specific hypertension and responsiveness to nitric oxide in sGCα1 knockout mice. *Cardiovasc Res*. 2008;79:179–186.
32. Sessa WC. eNOS at a glance. *J Cell Sci*. 2004;117:2427–2429.
33. Akimoto Y, Horinouchi T, Shibano M, et al. Nitirc oxide (NO) primarily accounts for endothelium-dependent component of β-adrenoceptor-activated smooth muscle relaxation of mouse aorta in response to isoprenaline. *J Smooth Muscle Res*. 2002;38:87–99.
34. Banquet S, Delannoy E, Agouni A, et al. Role of $G_{i/o}$-Src kinase-PI3K/Akt pathway and caveolin-1 in β_2- adrenoreceptor coupling to endothelial NO synthase in mouse pulmonary artery. *Cell Signal*. 2011;23:1136–1143.
35. Darsalia V, Larsson M, Lietzau G, et al. Gliptin-mediated neuroprotection against stroke requires chronic pretreatment and is independent of glucagon-like peptide-1 receptor. *Diabetes Obes Metab*. 2016;18:537–541.
36. Fleming I, Busse R. Signal transduction of eNOS activation. *Cardiovasc Res*. 1999;43:532–541.
37. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J*. 2012;33:829–837.
38. Graefe-Mody U, Retlich S, Friedrich C. Clinical pharmacokinetics and pharmacodynamics of linagliptin. *Clin Pharmacokinet*. 2012;51:411–427.
39. Hardigan T, Yasir A, Abdelsaid M, et al. Linagliptin treatment improves cerebrovascular function and remodeling and restores reduced cerebral perfusion in type 2 diabetes. *Am J Physiol Regul Integr Comp Physiol*. 2016, DOI: 10.1152/ajpregu.00057.2016. [Epub ahead of print].

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