ORIGINAL RESEARCH

Influence of type-4 dipeptidyl peptidase inhibition on endothelium-dependent relaxation of aortae from a db/db mouse model of type 2 diabetes: a comparison with the effect of glimepiride

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Purpose: The aim of this study was to investigate the effects of the type-4 dipeptidyl peptidase (DPP-4) inhibitors linagliptin and vildagliptin as well as the sulfonylurea glimepiride on endothelium-dependent relaxation of aortae from female db/db mice with established hyperglycemia to determine whether these treatments were able to attenuate diabetes-induced endothelial dysfunction.

Materials and methods: The mice were treated with glimepiride (2 mg/kg po per day, weeks 1–6, n=12), glimepiride plus vildagliptin (glimepiride 2 mg/kg po per day, weeks 1–6; vildagliptin 3 mg/kg po per day, weeks 4–6, n=11), glimepiride plus linagliptin (glimepiride 2 mg/kg po per day, weeks 1–6; linagliptin 3 mg/kg po per day, weeks 4–6, n=11) or linagliptin (3 mg/kg po per day, weeks 1–6, n=12). Endothelium-dependent relaxation using acetylcholine was assessed in the absence and presence of pharmacological tools (TRAM-34 1 μM; apamin 1 μM; N-nitro-L-arginine [L-NNA] 100 μM; 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one [ODQ] 10 μM) to distinguish relaxation mediated by nitric oxide (NO).

Results: Linagliptin was associated with a significant improvement in endothelium-dependent relaxation (ACh Rmax; db/db 41±1%, linagliptin 73±6%, $p<0.05$). The enhanced response was maintained in the presence of TRAM-34+ apamin (ACh Rmax; db/db 23±6%, linagliptin 60±6%, $p<0.01$), ie, when the endothelium-dependent relaxation was mediated by NO. There was no evidence for a contribution from K_{Ca} channel opening to responses under any conditions. Glimepiride had no effect on endothelium-dependent relaxation when given alone (ACh Rmax 38±3%). The addition of linagliptin or vildagliptin to glimepiride did not significantly improve endothelium-dependent relaxation. All treatments caused some decrease in aortic superoxide production but the effect of linagliptin was significantly greater than glimepiride (linagliptin 534±60 relative luminescence unit [RLU], glimepiride 1471±265 RLU, $p<0.05$).

Conclusion: Linagliptin is superior to glimepiride in regard to the preservation of endothelium-dependent relaxation in the presence of hyperglycemia and the improvement in endothelial function in response to linagliptin treatment is associated with greater antioxidant activity compared to glimepiride.

Keywords: diabetes, DPP-4 inhibitor, endothelium-dependent relaxation, glimepiride, linagliptin, reactive oxygen species

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Introduction

Type-4 dipeptidyl peptidase (DPP-4) inhibitors are used to attenuate hyperglycemia in type 2 diabetes. There is growing evidence that this class of drugs may have

additional effects on cardiovascular function independently of those on plasma glucose that may be viewed as beneficial.[1,2] We demonstrated previously that the DPP-4 inhibitor, linagliptin, exerts an acute antioxidant effect in vitro, a property not shared by two other DPP-4 inhibitors, vildagliptin and sitagliptin.[3] It has been shown that the DPP-4 inhibitor teneligliptin exerts antioxidant activity in human umbilical vein endothelial cells.[4] Linagliptin also improves endothelium-dependent relaxation of mesenteric arteries that have been impaired in the presence of high glucose-induced oxidative stress.[3] Furthermore, in rats with streptozotocin-induced type 1 diabetes, exposure to linagliptin appeared to block diabetes-induced impairment of endothelial function.[5] Notably, the effect on the endothelium occurred in the absence of any observable linagliptin-induced reduction in plasma glucose concentration. The apparent effect of linagliptin to improve endothelial function, by a mechanism that is independent of any action on glucose concentration, is supported by observations that linagliptin treatment improves impaired endothelial function in mice with elevated lipid levels or where endothelium-dependent relaxation was impaired by sodium arsenite-induced oxidative stress.[6] These findings are consistent with observations in diabetic patients, where DPP-4 inhibitors appear to improve endothelium-dependent relaxation in terms of increased flow-mediated dilatation of the brachial artery although it should be noted that not all studies with DPP-4 inhibitors have confirmed this effect.[7–9]

The sulfonylureas are another group of frequently used anti-diabetic agents. In contrast to DPP-4 inhibitors, there is no clinical evidence that sulfonylureas, such as glimepiride, have any direct effect on endothelial function[10–12] despite some pre-clinical evidence supporting increased endothelial nitric oxide synthase (eNOS) activity with glimepiride[13,14] and apparent antioxidant properties in patients with type 2 diabetes.[15] In the treatment of type 2 diabetes, the sulfonylureas and DPP-4 inhibitors may be used either alone or in combination[16] but to the best of our knowledge, there are no previous reports of the effect on endothelial function of the combined use of these compounds.

The aim of this in vitro study, performed in a db/db mouse model of type 2 diabetes, was to investigate how two DPP-4 inhibitors, linagliptin and vildagliptin, impact on production of reactive oxygen species (ROS) and endothelial function when administered in combination with the sulphonylurea, glimepiride. Further, given the ongoing CAROLINA (Cardiovascular outcome trial of linagliptin versus glimepiride in type 2 diabetes[17]) clinical trial, it was of interest to also directly compare the effects of glimepiride and linagliptin when given alone.

Materials and methods
Animals
Female db/db mice (aged 5–6 weeks) were assigned randomly to one of five active study groups where they received: no treatment (n=12); glimepiride alone from weeks 1–6 (n=12); glimepiride from weeks 1–6 with vildagliptin from weeks 4–6 (n=11); glimepiride from weeks 1–6 with linagliptin from weeks 4–6 (n=11); or linagliptin alone from weeks 1–6 (n=12). Doses employed were: glimepiride (2 mg/kg po per day); vildagliptin (3 mg/kg po per day); and linagliptin (3 mg/kg po per day). A sixth group of untreated C57/BL6 mice served as non-diabetic controls (n=10). The dose of glimepiride used has been shown to engender effective glucose control in mice fed a high-fat diet[18] and linagliptin and vildagliptin have been demonstrated to effectively lower plasma DPP-4 activity at 3 mg/kg/day.[19]

All animals underwent sampling for determination of plasma glucose concentration, type-1 glucagon-like peptide (GLP-1) and glycated hemoglobin (Hb_{A1c}) before treatment was commenced with blood obtained from the tail vein. The mice were not fasted prior to blood collection. Measurements were repeated at Week 3 and at the end of the period of experimentation, prior to euthanasia by carbon dioxide asphyxiation. Final blood samples were obtained from the left ventricle by cardiac puncture and glucose concentration measured using a one-touch glucometer (Roche, Sydney, NSW, Australia). Hb_{A1c} was measured by immunoassay using an A1cNow® kit (POCD, Sydney, NSW, Australia) and GLP-1 was measured using a commercial enzyme-linked immunosorbent assay (Merck Millipore, USA).

All procedures involved were approved by the Animal Experimentation Ethics Committees of RMIT University (AEC no. 1609) and conform to the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes.

Myograph experiments
Thoracic aortae were dissected and placed in Krebs bicarbonate solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 11.1 mmol/L D-glucose and 2.5 mmol/L $CaCl_2$,

pH 7.4), aerated with 5% carbon dioxide (CO_2) in oxygen (O_2) and maintained at 37°C. Fat and connective tissue were cleared from the arterial tissue, which was then cut into long ring segments (2–3 mm). Aortic rings were mounted in a myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) containing Krebs solution containing the cyclooxygenase inhibitor indomethacin (10 μM). After the rings were mounted, they were allowed to stabilize for a period of 15–30 mins while under a tension of 5 mN. All experiments were conducted at 37ºC in the Krebs solution aerated with carbogen (95% O_2 and 5% CO_2). Once resting tension had stabilized, the thromboxane receptor agonist U46619 (10 μM) was added for 20 mins to induce maximum contraction. To assess the integrity of the endothelium, the aortic rings were pre-contracted to ~50% of maximum response with the thromboxane receptor agonist U46619 (1–10 nM) and a single concentration of acetylcholine (10 μM) was used to relax the artery rings. The endothelium of individual ring preparations were considered to be functionally intact where acetylcholine-induced relaxation was greater than 70% of the pre-contracted tone. After further washouts and return to basal tension, arteries were again pre-contracted to ~50% of maximum response using the α-adrenoceptor agonist, cirazoline (0.01–0.1 μM). The effect of the treatment on relaxant responses was examined by constructing cumulative concentration-response curves to acetylcholine (0.1 nM–10 μM). In addition, responses to acetylcholine were examined after incubation for 20 mins with different combinations of: N-nitro-L-arginine (L-NNA, 100 μmol/L), a non-selective nitric oxide synthase inhibitor; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μmol/L), an inhibitor of soluble guanylate cyclase (sGC); 1-[(2-chlorophenyl) (diphenyl) methyl]-1H-pyrazole (TRAM-34, 1 μM), a selective inhibitor of the intermediate-conductance calcium-activated potassium channel (IK_{Ca}); and apamin (1 μM), a small-conductance calcium-activated potassium channel (SK_{Ca}) inhibitor.

Vascular superoxide

Production of superoxide anion in aortic rings was measured using L-012 chemiluminescence as described previously[20] with the following modifications. Aortic segments were cleared of fat and connective tissue and cut into segments (2–3 mm) which were incubated in cell culture plates at 37°C for 30 mins in Krebs-HEPES buffer.

A single aortic segment was added to each well of a 96-well optiplate with Krebs-HEPES buffer (300 mL),

containing L-012 (100 mM, Wako Pure Chemicals, Osaka, Japan) and the appropriate investigational treatments before loading into a Polarstar Optima plate reader (BMG Labtech, Melbourne, VIC, Australia) to measure photon emission at 37°C. Background readings without aortic segments were taken first. Superoxide production was quantified by subtracting the final reading from the background reading and counts were then taken as arbitrary units of superoxide production and expressed as a ratio to dry tissue mass (AU/mg dry tissue).

Drugs

All drugs were purchased from Sigma-Aldrich (Australia) except for linagliptin which was a gift from Boehringer-Ingelheim. Stock solutions (10 mg/mL) of glimepiride, linagliptin and vildagliptin were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. For administration by gavage, these drugs were further diluted in 1% w/v methylcellulose. L-NNA was dissolved in 0.1 M sodium bicarbonate, ODQ and TRAM-34 were dissolved in dimethyl sulfoxide and apamin was dissolved in distilled water.

Statistical analysis

Results were compared by one-way or two-way analysis of variance (ANOVA) with a post hoc Dunnett's or Sidak's test using Graphpad Prism (Version 7, GraphPad Software, San Diego, USA). Comparisons providing p-values of less than 0.05 were considered as indicating statistical significance.

Results

Body weight, blood glucose and Hb_{A1c}

Changes in mean body weight, blood glucose concentration and Hb_{A1c} in the six different groups over the 6-week study period are shown in Figure 1. Mean body weights of the db/db mice were significantly higher than that in the C57/BL6 mice (Figure 1A, $p<0.001$). All mean (SEM) body weights increased during the study but there was no significant impact of any of the investigatory agents on weight. Mean blood glucose concentration and Hb_{A1c} in the db/db mice were significantly higher than in the C57/BL6 mice over the course of the study ($p<0.001$ in both cases). Mean blood glucose levels and Hb_{A1c} increased in all db/db mice study groups over the 6-week study but there was no significant impact of any of the investigatory agents on either parameter (Figure 1B).
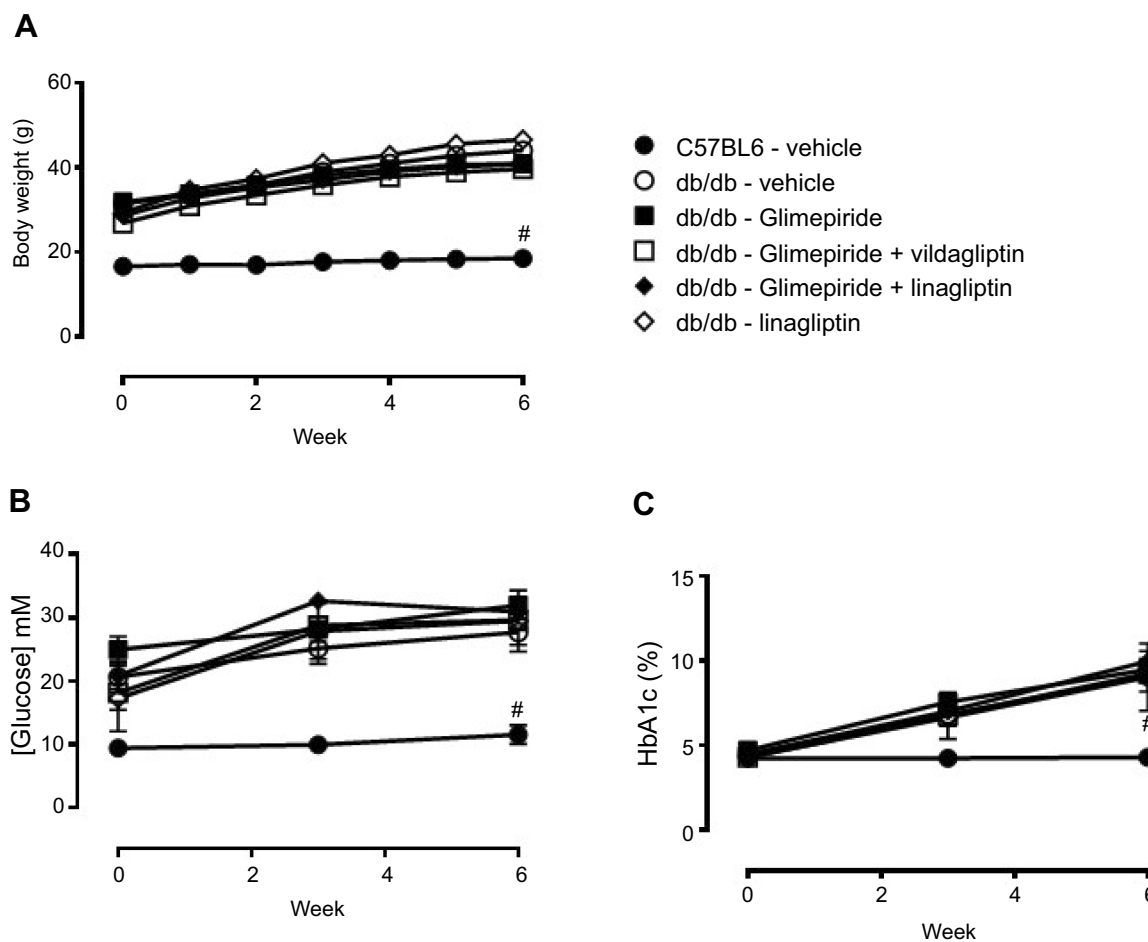
Figure 1 Body weights (**A**), blood glucose concentration (**B**) and HbA_{1c} (**C**) in the six different groups of mice over the 6-week treatment period. All parameters were significantly higher in db/db compared to C57/BL6 mice and none of the treatments had any effect. Data are expressed as means ± standard error (SEM). [#]$p<0.001$ compared to db/db over the 6-week treatment period, two-way ANOVA and Dunnett's test, n=10–12.
Abbreviation: ANOVA, analysis of variance.

During the study, mean blood glucose levels increased slightly whereas mean Hb_{A1c} levels remained stable in the C57/BL6 mice (Figure 1C). Mean plasma GLP-1 concentrations measured at the end of the 6-week study period were significantly higher in the vehicle-treated db/db mice compared to the vehicle-treated C57/BL6 mice (Figure 2). None of the treatments affected significantly the GLP-1 concentrations in db/db mice.

Vascular superoxide

Mean superoxide release from isolated aortic rings dissected from vehicle-treated db/db mice and measured using the L-012 luminescence assay was significantly higher than those from vehicle-treated C57/BL6 mice ($p<0.05$; Figure 3). Pretreatment with glimepiride in the db/db mice was associated with a significant reduction in mean aortic superoxide release (versus vehicle-treated db/db mice) that was similar in both the absence and presence of vildagliptin co-administration

($p<0.05$ in both cases). Pretreatment with linagliptin was also associated with a reduction in mean aortic superoxide release (versus vehicle-treated db/db mice; $p<0.05$). Linagliptin, either alone or with glimepiride, caused a significantly greater reduction in superoxide compared to glimepiride, either alone or with vildagliptin.

Myograph experiments

Acetylcholine concentration response (relaxation %) curves constructed for the aortae from animals from each of the six treatment groups are shown in Figures 4 and 5. Mean acetylcholine sensitivity (pEC_{50}) profile was similar in all six groups (Table 1). The mean maximum acetylcholine relaxation response in the db/db mice was significantly lower (approx. 50%) than that seen in the C57BL6 mice (Figure 4A). Mean maximum relaxation in aortic rings from db/db animals pretreated with glimepiride was similar to that in vehicle-treated db/db mice. In contrast, mean maximum relaxation was greater
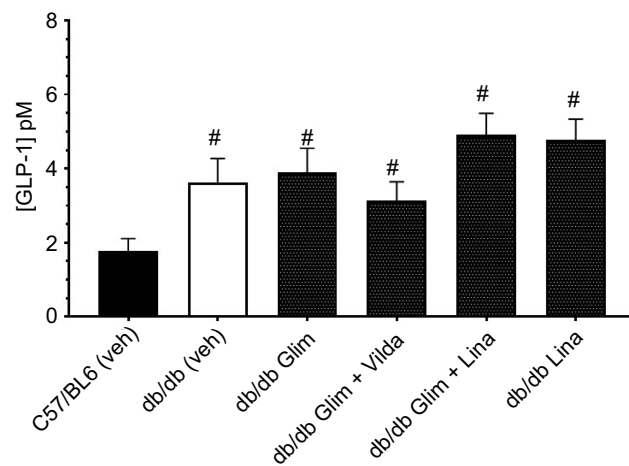
Figure 2 The plasma concentration of glucagon-like peptide 1 (GLP-1) was significantly lower in C57/BL6 compared to db/db mice. GLP-1 concentration was not affected by any of the treatments. Data are expressed as mean ± SEM. #Significantly different compared to C57/BL6 (veh), $p<0.05$ one-way ANOVA and Sidak's test, n=10–12.
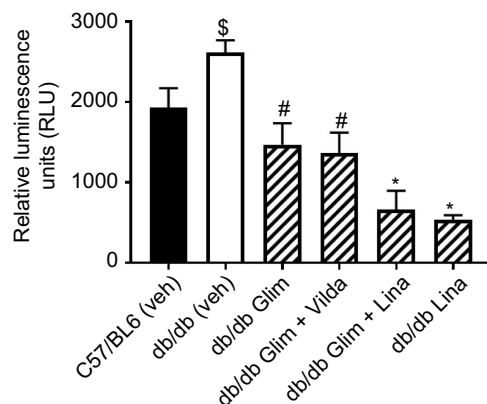**Abbreviation:** ANOVA, analysis of variance.

Figure 3 Measurement of superoxide generated in mouse aortae using L-012 enhanced chemiluminescence from the six groups. Data are expressed as mean ± SEM. **Notes:** $Significantly different to C57/BL6 (veh), $p<0.05$, one-way ANOVA, Sidak's multiple comparisons test. #Significantly different to db/db, $p<0.05$, one-way ANOVA, Sidak's multiple comparisons test. *Significantly different to db/db (veh) and db/db + glimepiride, $p<0.05$, one-way ANOVA, Sidak's multiple comparisons test. n=10–12.
Abbreviation: ANOVA, analysis of variance.

in animals treated with glimepiride in combination with either vildagliptin or linagliptin (Figure 5), though the differences from the vehicle-treated group did not achieve statistical significance (Table 1). However, mean maximum acetylcholine relaxation response in the db/db mice pre-treated with linagliptin alone was significantly higher than that in vehicle-treated db/db mice ($p<0.05$) and similar to that seen in the C57/BL6 mice (Figure 4A, Table 1).

The effect of the various treatments on the NO-mediated component of the endothelium-dependent aortic relaxation was investigated by constructing acetylcholine concentration
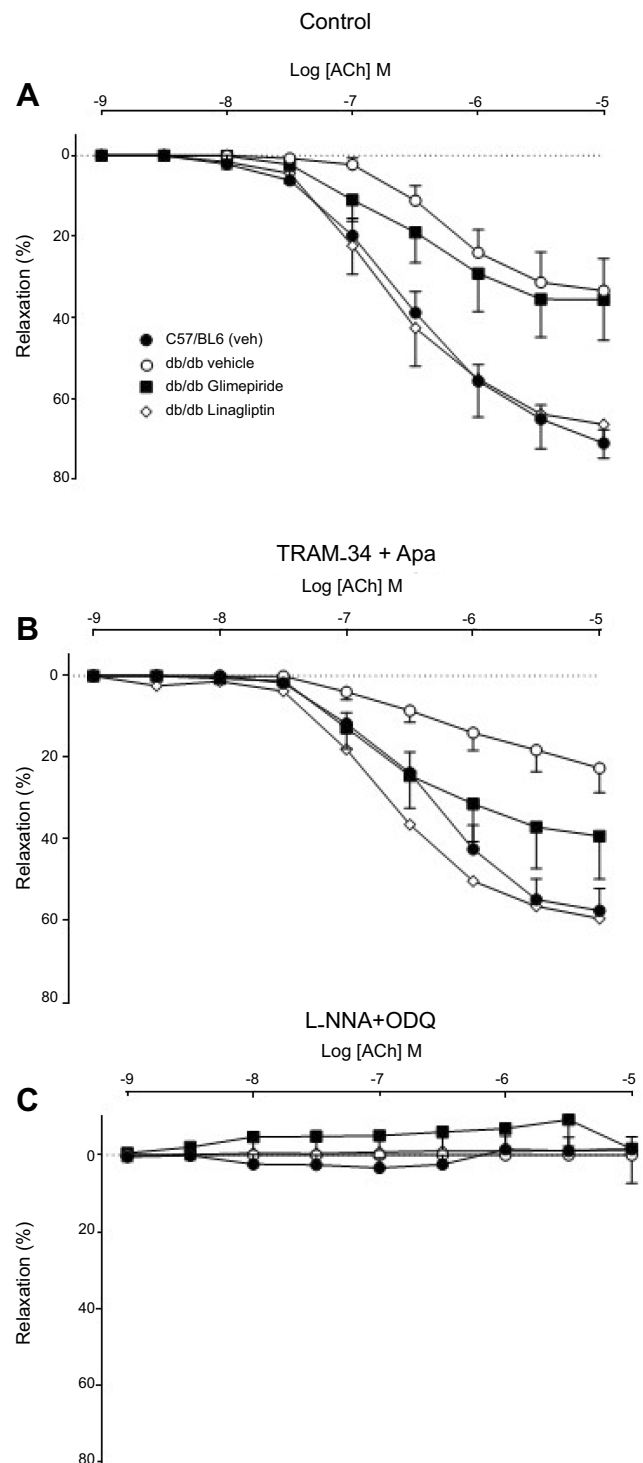

Figure 4 Concentration response curves to ACh aortae from C57/BL6 mice and db/db mice treated with vehicle, glimepiride or linagliptin for 6 weeks. Responses to ACh were tested in the absence (**A**) or presence of TRAM-34 plus apamin (**B**) to inhibit IK_{Ca} and SK_{Ca}, respectively, or N-nitro L-arginine (L-NNA) plus 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (**C**) to block nitric oxide synthase and guanylate cyclase, respectively. Calculated values for sensitivity (pEC_{50}) and maximum response (R_{max}) derived from these data are shown in Table 1.

response curves in the presence of TRAM-34 and apamin to inhibit IK_{Ca}- and SK_{Ca}-induced relaxation, respectively

A

Control
Log [ACh] M
(x-axis: -9, -8, -7, -6, -5)
y-axis: Relaxation (%) — 0, 20, 40, 60, 80

Legend:
● C57/BL6 (vehicle)
■ db/db Glimepiride
□ db/db Glimepiride + Vildagliptin
◆ db/db Glimepiride + Linagliption

B

TRAM_34 + Apa
Log [ACh] M
(x-axis: -9, -8, -7, -6, -5)
y-axis: Relaxation (%) — 0, 20, 40, 60, 80

C

L-NNA+ODQ
Log [ACh] M
(x-axis: -9, -8, -7, -6, -5)
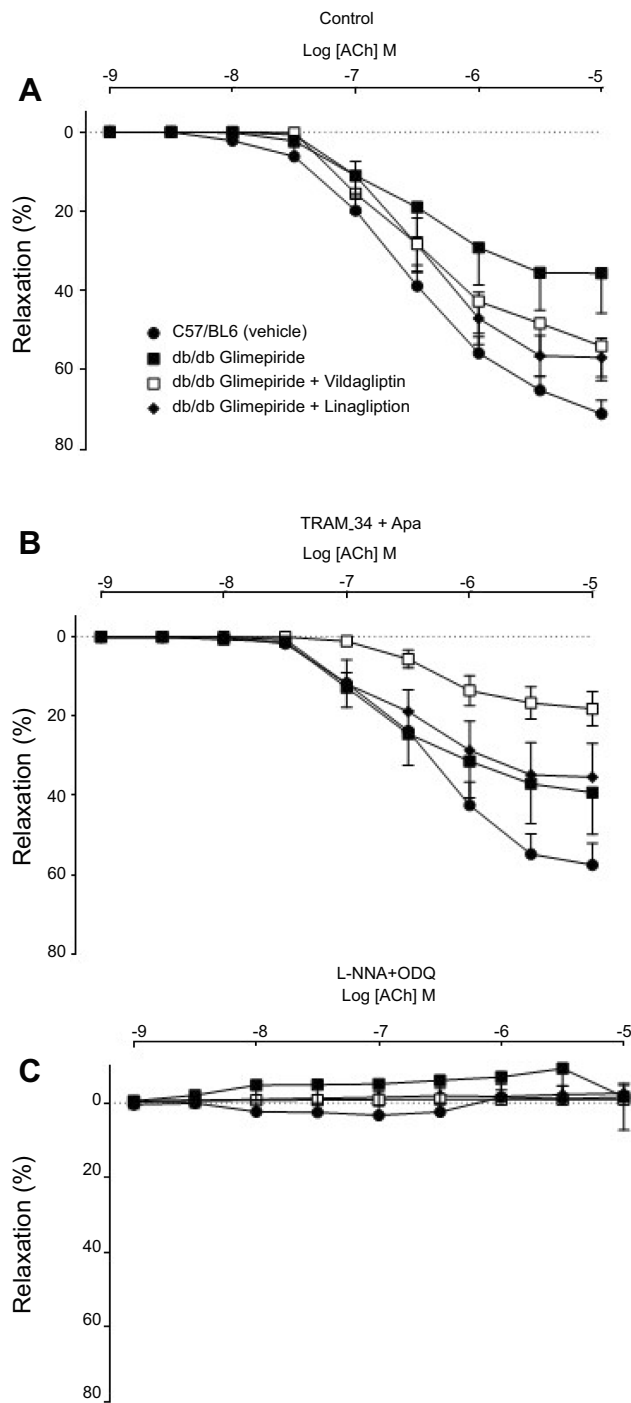y-axis: Relaxation (%) — 0, 20, 40, 60, 80

Figure 5 Concentration response curves to ACh in aortae from C57/BL6 mice and db/db mice treated with vehicle or glimepiride either alone or in combination with vildagliptin or linagliptin. Responses to ACh were tested in the absence (**A**) or presence of TRAM-34 plus apamin (**B**) to inhibit IK_{Ca} and SK_{Ca}, respectively, or N-nitro L-arginine (L-NNA) plus 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (**C**) to block nitric oxide synthase and guanylate cyclase, respectively. Calculated values for sensitivity (pEC50) and maximum response (Rmax) derived from these data are shown in Table 1.

Table 1 Sensitivity (pEC50) and maximum relaxant response (Rmax) to acetylcholine in aortic rings in the absence (control) and presence of TRAM-34 plus apamin derived from concentration response curves shown in Figures 4 and 5

Group	U46619 10^{-6} M (mN)	Cirazoline (% U46619)	ACh - Control pEC50 (M)	ACh - Control Rmax (%)	n	U46619 10^{-6} M (mN)	Cirazoline (% U46619)	ACh - TRAM-34+ Apamin pEC50 (M)	ACh - TRAM-34+ Apamin Rmax (%)	n
C57BL6 (+ vehicle)	17.3±1.6	58±7	6.58±0.11	71±3*	10	16.9±2.5	60±7	6.31±0.11	58±5#	10
db/db (+ vehicle)	18.4±2.5	46±4	6.08±0.12	41±1	12	17.0±2.5	47±6	6.23±0.26	23±6	12
db/db + Glimepiride	13.8±1.8	49±3	6.35±0.20	38±3	12	14.7±1.8	47±2	6.42±0.17	40±10	12
db/db + Glimepiride + Vildagliptin	15.8±3.3	55±10	6.47±0.13	53±8	11	16.6±3.2	47±7	6.25±0.12	24±4	11
db/db + Glimepiride + Linagliptin	21.1±2.4	47±4	6.44±0.13	57±5	11	23.1±0.9	40±2	6.50±0.15	36±8	11
	22.6±1.4	43±4	6.55±0.17	73±6*$	12	21.2±2.0	44±3	6.50±0.12	60±6#	9

Notes: There was no significant relaxant response to acetylcholine in the presence of L-NNA plus ODQ. The contractile responses to U46619 and cirazoline are also provided. *p<0.05 compared to db/db (+vehicle) under control conditions, Sidak's test. $ p<0.01 compared to db/db + Glimepiride under control conditions, Sidak's test. # p<0.01 compared to db/db plus apamin, Sidak's test. There were no significant differences in pEC50 or Rmax for ACh-induced relaxation between TRAM-34 plus apamin and control within any of the groups.

(Figures 4B and 5B). Acetylcholine-induced maximum relaxation in tissue from vehicle db/db mice was significantly lower than that seen in C57/BL6 mice whereas estimates of

sensitivity (pEC$_{50}$) were similar (Table 1). The acetylcholine response profile in the db/db mice exposed to glimepiride was similar to that of vehicle-treated db/db mice. In contrast, maximal aortic relaxation was significantly greater when the mice were treated with linagliptin alone (Table 1). The addition of linagliptin or vildagliptin to glimepiride did not significantly affect the response to acetylcholine. Acetylcholine-induced relaxation was abolished across the groups by incubation of aortic tissue with L-NNA and ODQ, added to inhibit nitric oxide synthase (NOS) and soluble guanylate cyclase, respectively (Figures 4C and 5C).

Discussion

Our in vivo study demonstrated an effect of linagliptin treatment (3 mg/kg po per day, 6 weeks) in female db/db mice on acetylcholine-induced endothelium-dependent relaxation of isolated thoracic aorta. Exposure to linagliptin appeared to redress the difference between these and the control C57/BL6 mice that has been reported previously.[21,22] The apparent effect of linagliptin on relaxation was markedly different from that of the sulfonylurea glimepiride administered under similar conditions. The differences in endothelium-dependent relaxation were less marked when mice were pretreated with linagliptin, or a second DPP-4 inhibitor vildagliptin, for a shorter period (3 weeks) in combination with glimepiride (6 weeks) and failed to achieve statistical significance versus relaxation in vehicle-treated control db/db mice. Comparison of the different acetylcholine-induced endothelium-dependent relaxation profiles in the presence of selective inhibitors of eNOS, guanylate cyclase, SK$_{Ca}$ and IK$_{Ca}$ channels indicated that the impact of linagliptin on endothelium-dependent relaxation was mediated through an effect on NO activity that occurs in the absence of any effect on endothelium-dependent hyperpolarisation.

In comparison to the control mice, the female db/db mice used in this study had significantly greater body weight, plasma glucose, HbA1c and plasma GLP-1 as expected in this model of type 2 diabetes. Interestingly none of the treatments, ie, glimepiride, DPP-4 inhibitors or their combination, had any effect on these parameters. This is similar to a previous report from Sharkovska et al[23] who found that linagliptin at the same dose failed to improve glucose control in male db/db mice. The dose of linagliptin was considered adequate as that dose has been shown previously to be equally effective as a 10-fold higher dose in the inhibition of DPP-4 activity.[24] Nagakura et al[25] found that whereas DPP-4 inhibitors were effective in young (6 weeks) db/db mice, they lost efficacy as the mice aged indicating that, as in the present study, in this model of β cell failure and high insulin resistance, the treatments did not effectively improve glucose control and our study suggests this is so for females as well as males. Despite this, the DPP-4 inhibitors did show evidence of improved endothelial function as we have previously reported in a model of type 1 diabetes.[5]

The capacity of linagliptin to redress the deficient endothelium-dependent relaxation reported in db/db mice appeared to be associated with a significant reduction in the generation of superoxide by the aortae. Vascular superoxide production was significantly elevated in aortae from the db/db mice compared to the control C57/BL6 mice, an observation that was consistent with other reports in a variety of animal models of both type 1 and type 2 diabetes.[26,27] We previously reported how treatment with linagliptin for 6 weeks significantly reduces vascular superoxide production in a model of type 1 diabetes (ie, streptozotocin-treated rats).[5] In this case, superoxide levels were also lower when mice were treated with linagliptin for a shorter period (3 weeks) in combination with glimepiride than those in db/db mice treated with vehicle or glimepiride alone. The apparent antioxidant activity of linagliptin is consistent with previously reported attenuation of superoxide levels in rat-isolated mesenteric arteries, both under control conditions and in the presence of high glucose concentrations (40 mM).[3] These findings suggest that linagliptin may scavenge oxygen radicals. This property may not be a group DPP-4 inhibitor effect, as in our previous study we found an absence of effect by vildagliptin or sitagliptin.[3] In the present study, the antioxidant action of vidagliptin plus glimepiride was not different to glimepiride alone and was significantly less than linagliptin with or without glimepiride. It seems likely also that the acute in vivo antioxidant activity of linagliptin is through a reduction, at least in part, of superoxide synthesis since it was associated with increased expression of eNOS as a dimer, which in its coupled form favors synthesis of NO rather than superoxide.[5] Linagliptin was also associated with a significant decrease in the expression of NOX2, another important source of vascular superoxide.[28] The evidence implies therefore that linagliptin serves to reduce both the synthesis and activity of superoxide, two possible mechanisms that might improve endothelium-derived NO activity. In contrast, glimepiride alone or in combination with vildagliptin pretreatment impacted significantly less on the diabetes-induced elevation in superoxide.

It is well known that the activity of endothelium-derived nitric oxide is impaired by oxidative stress, including that caused by diabetes. In the present study, which uses mouse thoracic aorta, the evidence points to the endothelium-dependent relaxation being mediated entirely by nitric oxide as the responses to acetylcholine were abolished by the combined inhibition of NOS by L-NNA and guanylate cyclase by ODQ. The findings suggest that NO-mediated relaxation is impaired in arteries from the diabetic db/db mice and that normalization of the relaxation response by linagliptin is maintained in the presence of TRAM-34 and apamin, inhibitors of IK_{Ca} and SK_{Ca}, respectively, which inhibit endothelium-dependent hyperpolarisation. It is worth noting that evidence from experiments conducted in the small mesenteric arteries taken from streptozotocin-treated rats suggests that linagliptin may also enhance relaxation through hyperpolarisation as well as NO-mediated mechanisms[5] whereas in this study using a conductance artery we found that TRAM-34 plus apamin had no effect on ACh-induced relaxation indicating no contribution of hyperpolarization to endothelium-dependent relaxation. Whilst we have previously found that a contribution of potassium channel opening does contribute to endothelium-dependent relaxation of rat aortae in a model of type 1 diabetes,[29] that was determined not to be the case in this type 2 diabetes model in mice.

It is noteworthy that our findings imply that the effect of linagliptin on aortic relaxation is independent of its actions on glucose metabolism. In the present work, linagliptin did not impact on blood concentrations of glucose or hemoglobin Hb_{A1c}, neither did it impact on body weight, as has been observed previously in type 1 diabetic rats.[3] Similarly, it has been reported previously how linagliptin in non-obese diabetic mice, another model of type 1 diabetes, resulted in an improvement of endothelium-dependent relaxation, an effect that was accompanied by an increase in eNOS expression and a decrease in expression of caveolin-1,[30] which inhibits eNOS activity. Previously, we have shown that linagliptin-induced improvement in endothelium-dependent relaxation is also accompanied by an increase in eNOS expression in mesenteric arteries from streptozotocin-treated rats. Taken together, the evidence appears to point to linagliptin possessing some property that enhances the synthesis of nitric oxide that is independent of any action it has on glucose homeostasis. Several clinical studies have made observations that suggest changes in endothelium-dependent relaxation in response to linagliptin. For example, researchers noted improvements in brachial artery flow-mediated dilatation (FMD) compared to metformin treatment alone when linagliptin was administered in addition to metformin to patients with type 2 diabetes.[31] It has been suggested that the effect on relaxation may be a DPP-4 class effect as both vildagliptin and sitagliptin have been shown to enhance endothelium-dependent relaxation in subjects with hyperglycemia.[32,33] However, any relationship appears to not be straightforward. Treatment with linagliptin alone was reported not to affect FMD despite in one report being associated with improvements in microvascular function that were independent of any changes in glucose regulation.[10] There are also reports in patients with type 2 diabetes where sitagliptin had no effect on FMD,[7] and in another case even caused a deterioration in endothelial function.

Evidence from studies conducted with linagliptin in conditions where endothelial dysfunction is not caused by hyperglycemia seems to support the potential for linagliptin to have beneficial vascular actions, again independent of glucose homeostasis. For example, linagliptin improved endothelial dysfunction in aortae from rats exposed to sepsis[34] when oxidative stress was increased by administration of sodium arsenite.[6] Linagliptin was also seen to improve endothelium-dependent relaxation in aortae from apolipoprotein-E-deficient mice, an effect that was accompanied by a decrease in oxidative stress and decreased development of atherosclerotic plaques.[35] These observations indicate vasculoprotective actions of linagliptin that extend to other diseases involving oxidative stress, such as atherosclerosis.

In conclusion, unlike glimepiride, linagliptin displays vasculoprotective properties that result, at least in part, from its antioxidant activity. This property may be common to DPP-4 inhibitors but evidence from this and our earlier study[5] suggests that linagliptin appears to be more effective at preserving endothelial function and reducing superoxide in comparison to other DPP-4 inhibitors.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

TK is an employee of Boehringer Ingelheim. OLW was contracted by Boehringer Ingelheim for conduct of research, during the conduct of the study. The authors report no other conflicts of interest in this work.

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