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Diabetes-mediated middle cerebral artery remodeling is restored by linagliptin: Interaction with the vascular smooth muscle cell endothelin system

Abdul Yasir^{1,2}, Trevor Hardigan², and Advije Ergul^{1,2}

¹Charlie Norwood Veterans Administration Medical Center, Augusta University, Augusta, Georgia

²Department of Physiology, Augusta University, Augusta, Georgia

Abstract

Introduction—Endothelin-1 (ET-1) mediates cerebrovascular remodeling in vascular smooth muscle layer of the middle cerebral arteries (MCA) in type-2 diabetic Goto-Kakizaki (GK) rats. While metformin, oral glucose lowering agent, prevent/restores vascular remodeling and reduce systemic and local ET-1 levels whether this effect was specific to metformin remained unknown. Our working hypotheses were 1) linagliptin, a DPP-IV inhibitor, can reverse diabetes-mediated cerebrovascular remodeling and this is associated with decreased ET-1, and 2) linagliptin prevents the high glucose induced increase in ET-1 and ET receptors in brain vascular smooth muscle cells (bVSMCs).

Methods—Diabetic and non-diabetic GK rats were treated with linagliptin (4 weeks). MCAs were fixed in buffered 4% paraformaldehyde and used for morphometry. Human bVSMCs incubated in normal glucose (5.5mM)/high glucose (25 mM) conditions were treated with the linagliptin (100nM; 24 hours). ET-1 secretion and ET receptors were measured in media and cell lysate respectively. Immunostaining was performed for ETA and ET-B receptor. ET receptors were also measured in cells treated with ET-1 (100nM) and linagliptin.

Results—Linagliptin treatment regressed vascular remodeling of MCAs in diabetic animals but had no effect on blood glucose. bVSMCs in normal/high glucose condition did not show any significant difference in ET-1 secretion or ET-A and ET-B receptor expression. ET-1 treatment in high glucose condition significantly increased the ET-A receptors and this effect was inhibited by linagliptin.

Conclusions—Linagliptin is effective in reversing established pathological cerebrovascular remodeling associated with diabetes. Attenuation of the ET system could be a pleiotropic effect of linagliptin that provides vascular protection.

Corresponding Author: Advije Ergul, MD, PhD, Department of Physiology, Augusta University, 1120 15th Street CA-2094, Augusta, Georgia 30912, aergul@gru.edu, Tel: 706.721.9103, Fax: 706.721.7299.

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Keywords

Diabetes; endothelin; linagliptin; brain vascular smooth muscle cells

Introduction

Diabetes induces a state of chronic inflammation and increases the risk of cerebrovascular diseases (1–4). It is well established that ET-1 contributes to diabetes-mediated vascular dysfunction and remodeling in multiple vascular beds including the cerebrovasculature (5–12). The binding of ET-1 with ET-A receptors on vascular smooth muscle cells (VSMC) promotes vasoconstriction and cell proliferation. The action of ET-B receptor differs with respect to its location; in endothelial cells ET-B receptors exert vasorelaxation to counter balance the response of ET-A receptors, whereas in VSMCs it exerts vasoconstriction similar to that of ET-A receptors (13; 14). Our group reported that either single or combined blockade of ET-A and ET-B receptors prevents cerebrovascular dysfunction and remodeling in the GK model of diabetes (5; 10; 11). Specifically, we showed that there is an increase in ET-B receptors on the VSM layer in our diabetic model (11). More recently we showed that inhibition of ET receptors with bosentan or glycemic control by metformin treatment reverses cerebrovascular remodeling and improves myogenic tone of large cerebral arteries (5; 15). The finding that glycemic control with metformin was also associated with decreased plasma ET-1 level and ET-A levels in the mesenteric resistance vessels suggested an interaction between glycemic control and ET system in diabetes (15). It is also possible that metformin has a direct effect. Recent evidence suggest that DPP-IV inhibitors, a new class of oral glucose lowering agents that is increasingly used in the management of type 2 diabetes (16), also improve endothelial dysfunction and reduce pro-oxidative and pro-inflammatory conditions independent of its glucose lowering effects (17). However, the effect of DPP-IV inhibitors on the ET system remained unknown. Thus, the current study was designed to determine the interaction of linagliptin, a DPP-IV inhibitor, with the ET system *in vivo* in diabetic GK rats and *in vitro* in bVSMC culture model. We hypothesized that linagliptin treatment can reverse diabetes-mediated cerebrovascular remodeling and this is associated with decreased ET-1. We further hypothesized that linagliptin prevents the high glucose induced increase in ET-1 secretion and upregulation of ET receptors in bVSMCs.

Materials and Methods

Animals and Drug Treatment

All experiments were performed on male GK (Tampa Colony, Taconic; Hudson, NY) rats. The animals were housed at the Augusta University animal care facility that is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the institutional animal care and use committee. Animals were fed standard rat chow and tap water *ad libitum*, and were maintained at 12 h light/dark cycle. Blood glucose levels were measured bi-weekly from tail vein samples using a commercially available glucometer (Freestyle, Abbott Diabetes Care, Inc; Alameda, CA). Glycosylated hemoglobin values (A1CNow-plus, PTS Diagnostics, Indianapolis, IN) were used as a measurement of long-term blood glucose levels. Rats were initially placed into two groups: those that did not

spontaneously develop hyperglycemia (HA1C% ≥ 7.0) and those that did develop hyperglycemia (HA1C% ≥ 7.0) by 14 weeks of age, which is past the age where GK rats have been shown develop hyperglycemia. All of the GK rats were litter controlled and fed the same diet under the same environmental conditions prior to the experimental treatment, and thus the nondiabetic GK rats were used as a genetically matched control for the diabetic GK rats in this study on the effects of glycemic control. At 24 weeks of age, after vascular disease is established in the diabetic group, the treatment with linagliptin was initiated in both control and diabetic rats (166 mg/kg in chow for 4 weeks). Animals were anesthetized by sodium pentobarbital and euthanized via cardiac puncture to isolate middle cerebral artery (MCA).

Vessel Morphometry and Immunohistochemistry

After sacrifice, MCA was isolated and mounted on arteriograph (Living Systems Instrumentations, Burlington, VT). After equilibration, vessels were pressure fixed in 4% paraformaldehyde buffer for morphometry. For morphometric analysis and immunohistochemistry, paraffin embedded 4–6 micron thick vessel cross sections were stained with Masson's trichrome stain or anti-ET-1 antibody, respectively. Slides were imaged using Axiovert microscope (Carl Zeiss Inc., Thornwood, NY) and wall thickness, lumen space were measured and media to lumen ratio were calculated (5; 11).

In Vitro bVSMCs Study

Human bVSMCs were procured from ScienCell research laboratories (Carlsbad, CA). Cells were grown in commercially available normal glucose (5.5 mM, Cat# 10-014-CM; 1g/L) and high glucose (25 mM, Cat# 10-013-CV; 4.5g/L) DMEM media (Corning, Cellgro, Manassas, VA 20109). Both media used in the current study had similar osmolality of 335 ± 30 mOsm/kg H_2O . Cells were incubated in normal glucose (5.5mM) or high glucose (25mM) media for 24 hours with and without DPP-IV inhibitor linagliptin (100nM). Cell media was collected for ET-1 measurement by a commercially available ELISA kit (Biotek, R&D, USA). In a separate set of experiments, cells were challenged with ET-1 (100nM) in normal and high glucose conditions. Cell lysate was prepared for the estimation of ET-A and ET-B receptor by Western blotting. Briefly, equivalent amounts of cell lysates of human bVSMCs (15 μ g protein/lane) were loaded onto 10% SDS-PAGE, proteins separated, and proteins transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin followed by incubation for 12 hours at 4°C with appropriate primary antibodies. ET-A (Abcam; cat# ab85163) and ET-B (Alomone labs; cat # AER-002) at 1:1000 dilutions or b-actin at 1:3000 dilutions were used. After washing, membranes were incubated for 1 hour at 20°C with appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated; dilution 1:3000). Prestained molecular weight markers were run in parallel to identify the molecular weight of proteins of interest. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent and the signals were monitored on Alpha Imager (Alpha Innotech; (San Leandro, CA). Relative band intensity was determined by densitometry software (Alpha Innotech, ProteinSimple, San Jose, CA) and normalized with b-actin protein. Fluorescent immunostaining for ET-A and ET-B was performed on cells grown on the slides. Slides were imaged on Axiovert 200 microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Data Analysis

Two-way ANOVA (2×2 design) was used to assess disease and treatment effects in GK rats and first set of cell culture studies (Control vs diabetes or normal glucose vs high glucose X Linagliptin yes or no). A Bonferroni's post-test adjustment for multiple comparisons was used for all post-hoc mean comparisons for significant effects from all analyses. For cell culture studies in which exogenous ET-1 was added, one-way ANOVA was used to compare vehicle, ET-1 and ET-1 + linagliptin under normal or high glucose conditions followed by a Tukey's post-hoc comparison. Data was expressed as Mean $\pm$ SEM and $p < 0.05$ was considered significant.

Results

MCA Morphology and ET-1 Immunostaining

At 28 weeks of age, diabetic animals exhibited increased wall thickness and media to lumen ratio in MCAs (Fig 1B & D). There was a disease and treatment interaction such that these indices were restored in diabetic but not in control animals treated with linagliptin. This effect was independent of blood glucose because linagliptin did not lower blood glucose in diabetic rats (97 ± 3 vs 193 ± 40 mg/dl in control and diabetic animals treated with linagliptin, respectively). There was prominent ET-1 staining in both endothelial and vascular smooth muscle layers in diabetes. Linagliptin-treated diabetic rats showed relatively less staining localized mostly to endothelial layer (Fig 1A)).

Effect of High Glucose and Linagliptin on bVSMC ET System

High glucose culture conditions did not impact ET-A receptor levels. Similarly, there was no effect of high glucose and/or linagliptin on ET-B receptors or ET-1 levels (Fig. 2A–C). Immunohistochemical localization studies in intact bVSMCs grown under normal and high glucose conditions yielded similar results. ET-A or ET-B receptor expression in bVSMCs was not different between normal glucose, high glucose and linagliptin treated cells (Fig. 3A and B). However, there was a strong perinuclear staining in all groups.

Effect of ET-1 on ET-A and ET-B Receptors in Normal and High Glucose Conditions

Stimulation of bVSMCs with exogenous ET-1 under normal glucose containing medium did not have any effect on ET-A or ET-B receptors (Fig. 4A and B). On the other hand when bVSMCs were challenged with ET-1 in high glucose growth conditions, ET-A, but not ET-B, receptor was significantly increased and this effect was prevented by linagliptin co-treatment (Fig. 4C–D).

Discussion

Our previous findings that 1) diabetes-mediated cerebrovascular remodeling is associated with increases in VSM layer ET-B expression, and 2) both glycemic control and ET receptor antagonism prevents/restores this pathological remodeling have led us to the main questions of this study: Can an oral hypoglycemic drug that restores hypertrophic remodeling of the cerebral vasculature regulate the bVSMC ET system and if so, is this effect glucose-dependent? Our results show that treatment of diabetic GK rats with linagliptin after

established vascular disease restores MCA structure. This is associated with decreased ET-1 staining in the VSM layer and independent of blood glucose. On the other hand, high glucose growth conditions do not increase ET-1 secretion or ET receptor expression in bVSMCs in our experimental design. Treatment of bVSMCs with ET-1 under high glucose conditions upregulates ET-A receptor levels and this effect is blunted by linagliptin.

Due to its potent proliferative, proinflammatory and profibrotic actions, ET-1 has been implicated to contribute to pathological remodeling of the vasculature in a number of disease states including hypertension and diabetes (1; 18–20). Our group has shown that diabetes mediates hypertrophic remodeling of MCAs (10; 11) and mesenteric resistance vessels (12; 15; 21). As discussed in greater detail below, ET receptor antagonism prevented and reversed this pathological remodeling in a vascular bed specific manner (5; 9–11; 21). Glycemic control achieved by metformin was also able to prevent and reverse vascular remodeling on both vascular beds (15; 22) and decreased plasma and tissue ET-1 levels. Given that glycemic control remains to be a key treatment strategy for the management of vascular complications of diabetes and most patients come to the clinic after onset of these complications, we were interested in testing whether linagliptin, a DPP-IV inhibitor class of oral hypoglycemic that is increasingly used for treatment of diabetes, modulates the ET system after vascular disease is established (16). The results of this study demonstrate that linagliptin partially restores the structure of MCAs while reducing ET-1 levels without any change in blood glucose levels, which led us to further investigate the regulation of ET receptors by linagliptin in an *in vitro* model.

ET-1 is a potent endogenous vasoconstrictor mainly secreted by endothelial cells however, now it is evident that it can be produced by other cell types also including VSMCs (23–25). Expression of ET-A and ET-B receptors on VSMCs mediates vasoconstriction and proliferation, whereas, ET-B receptors located on endothelial cells mediate vascular relaxation and considered to be vasculoprotective (14; 26). We have shown that ET-A and dual ET-A/ET-B antagonism prevents diabetes-mediated remodeling of the mesenteric vessels that is characterized by augmented collagen deposition and increased wall thickness (21; 27). On the other hand, selective ET-B blockade worsens vascular remodeling, providing support to vasculoprotective effects of this receptor subtype (21). In a follow-up study, we hypothesized that diabetes down regulates vasculoprotective endothelial ET-B receptors in the cerebrovasculature and selective blockade of this receptor subtype would amplify diabetes-mediated MCA remodeling. In contrast to our hypothesis, selective ET-B blockade yielded similar results with selective ET-A or dual receptor antagonism and prevented remodeling (10; 11). This effect was most likely due to upregulation of ET-B receptors on VSMCs. In the current study, ET-1 staining was observed in both endothelial and VSM layers in untreated diabetic animals and linagliptin decreased staining intensity in VSM layer. Thus, we further investigated the changes in the ET system in bVSMC grown under normal and high glucose conditions. Incubation of human bVSMCs with high glucose containing media did not affect ET-1 or ET receptor levels. Interestingly, in high glucose conditions, there was significant perinuclear staining for ET receptors. Avedanian *et al.* (28) reported similar staining pattern in human aortic endothelial cells and we reported increased perinuclear endothelin converting enzyme staining in endothelial cells grown in high glucose conditions (29). As recently reviewed, nuclear ET receptor localization and signaling has

also been reported in cardiac myocytes (30). Linagliptin treatment did not affect ET-1 production or ET receptor expression. While the above observations suggest that high glucose and linagliptin treatment did not alter bVSMCs ET system, we also consider the limitations of the experiments performed. The duration of study (24 hours) and high glucose (25mM) used in the present study may not be sufficient to activate ET system of VSMCs. High glucose stimulates endothelial ET-1 secretion which can then act upon underlying smooth cells. To mimic this possibility, bVSMCs were challenged with ET-1 in normal/high glucose containing media and treated with linagliptin. Interestingly, incubation with ET-1 increased the expression of ET-A receptors in high glucose containing media and linagliptin prevented this effect, suggesting the vasculoprotective effect we observed with linagliptin in diabetic animals may be due to the down regulation of this receptor subtype, which needs to be further pursued in future studies.

Glucose lowering effects of DPP-IV inhibitors are based on the inhibition of degradation of glucagon like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) by DPP-IV. However, there is increasing evidence for pleiotropic actions of DPP-IV and DPP-IV inhibitors (31). The vasculoprotective effects of these drugs are not fully understood in part due to the wide range of DPP-IV substrates in addition to GLP-1 and GIP. Examples include neuropeptides, hormones, and meprin- β , which has a role in activation of pro-inflammatory cytokines like TNF- α , IL-1 β , TGF β . It is known that ET-1 contributes in inflammatory processes in the vascular wall involving activation of NF- κ B and pro-inflammatory cytokines including TNF- α , IL-6, IL-1 (32) and these pro-inflammatory cytokines in turn stimulate ET system (33). Thus, the inhibition of pro-inflammatory pathways via DPP-IV inhibition by linagliptin could have contributed to reduction of ET-1 in the vascular wall, which remains to be determined. Further, in diabetic animal model, DPP-IV inhibition increases the bio-availability of GLP-1 for its binding to GLP-1R receptors. In a related study we determined that DPP-IV activity is significantly decreased with our treatment paradigm but we did not measure GLP-1 levels, which can decrease VSMC growth directly (31). This class of agents can also increase eNOS activity in isolated endothelial cells as well as in Zucker obese rats and spontaneously hypertensive rats (34; 35). Salheen and colleagues showed that linagliptin is effective in improving endothelium-dependent relaxation of mesenteric vessels incubated in high glucose as short as two hours due to its antioxidant effects (36). Collectively, these reports suggest that regulation of endothelial eNOS as well as systemic GLP-1 and inflammatory cytokines may have contributed to vasculoprotective effects observed in diabetic rats. However, our finding that linagliptin prevents high glucose-mediated upregulation of ET-A receptors on bVSMC also suggests that linagliptin has independent effects on these cells via a mechanism we do not completely understand.

In conclusion, current study provides novel information that DPP-IV inhibitor linagliptin can reverse cerebrovascular remodeling in diabetic animals. Inhibition of the ET system could be one of the pleiotropic effects exerted by linagliptin that contributes to its vasculoprotective properties. These results suggest that linagliptin treatment can be used as therapeutic strategy in diabetes to prevent vascular complications.

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Figure 1. Linagliptin restores cerebrovascular structure in diabetes. **A)** Representative images of cross sections of MCAs stained by Masson's trichrome staining or anti-ET-1 antibody. **B), C)** and **D),** Measurement of wall thickness, lumen diameter and media to lumen ratio respectively. # $p=0.0145$; ## $p=0.0001$ disease and treatment interaction. * $p<0.001$ Compared with diabetic vehicle treated group. Results are expressed as mean $\pm$ SEM, (n=4).

Figure 2.

High glucose and linagliptin treatment has no effect on endothelin (ET) system of bVSMCs. **A)** and **B)** representative immunoblots and analysis of ET-A and ETB receptors respectively in bMVSCs incubated in normal glucose (5mM)/high glucose (25mM) conditions for 24 hours and treated with linagliptin (100nM) did not show any difference in expression of ET-A and ET-B receptors. **C)** Level of secreted ET-1 in media measured by ELISA was not changed with high glucose and linagliptin treatment. Results are expressed as mean $\pm$ SEM, (n=4).

Figure 3.

High glucose and linagliptin treatment has no effect on immunostaining for ETA and ETB receptor in bVSMCs. **A)** Perinuclear expression of ETA receptor in bMVSCs was observed however, it was not different in normal glucose (5mM)/high glucose (25mM) conditions and linagliptin treatment for 24 hours (Scale bar is 50 μ m; n=3). **B)** Perinuclear expression of ETB receptor was observed in normal (5mM) as well as high glucose (25mM) conditions for 24 hours. Linagliptin (100nM) treatment did not change the expression of ET-B receptor (Scale bar is 30 μ m; n=3).

Figure 4.

Endothelin-1 (ET-1) upregulates ET-A receptor expression in high glucose condition and this effect is blunted by linagliptin in bVSMCs. **A) – D)** representative immunoblots and analysis of ET-A and ET-B receptors expression in bVSMCs after incubation with ET-1 for 24 hours in normal/high glucose conditions with or without linagliptin treatment. **A)** and **B)** ET-1 (100nM) has no effect on expression of ET-A and ET-B receptors in normal glucose (5mM) condition. Linagliptin treatment also did not change the expression of these receptors. **C)** ET-1 significantly increased the expression of ET-A receptor in high glucose (5mM) condition ($p < 0.05$; compared with high glucose alone) and linagliptin treatment blunted this effect. **D)** ET-1 and linagliptin treatment did not change the expression of ET-B receptor in high glucose condition. Results are expressed as mean $\pm$ SEM, (n=3).