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Linagliptin reduces effects of ET-1 and TLR2-mediated cerebrovascular hyperreactivity in diabetes

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ABSTRACT

Aims: The anti-hyperglycemic agent linagliptin, a dipeptidyl peptidase-4 inhibitor, has been shown to reduce inflammation and improve endothelial cell function. In this study, we hypothesized that DPP-IV inhibition with linagliptin would improve impaired cerebral blood flow in diabetic rats through improved insulin-induced cerebrovascular relaxation and reversal of pathological cerebrovascular remodeling that subsequently leads to improvement of cognitive function.

Main methods: Male type-2 diabetic Goto-Kakizaki (GK) and nondiabetic Wistar rats were treated with linagliptin, and ET-1 plasma levels and dose response curves to ET-1 (0.1–100 nM) in basilar arteries were assessed. The impact of TLR2 antagonism on ET-1 mediated basilar contraction and endothelium-dependent relaxation to acetylcholine (ACh, 1 nM–1 M) in diabetic GK rats was examined with antibody directed against the TLR2 receptor (Santa Cruz, 5 μg/mL). The expression of TLR2 in middle cerebral arteries (MCAs) from treated rats and in brain microvascular endothelial cells (BMVEC) treated with 100 nM linagliptin was assessed.

Key findings: Linagliptin lowered plasma ET-1 levels in diabetes, and reduced ET-1-induced vascular contraction. TLR2 antagonism in diabetic basilar arteries reduced ET-1-mediated cerebrovascular dysfunction and improved endothelium-dependent vasorelaxation. Linagliptin treatment in the BMVEC was able to reduce TLR2 expression in cells from both diabetic and nondiabetic rats.

Conclusions: These results suggest that inhibition of DPPIV using linagliptin improves the ET-1-mediated cerebrovascular dysfunction observed in diabetes through a reduction in ET-1 plasma levels and reduced cerebrovascular hyperreactivity. This effect is potentially a result of linagliptin causing a decrease in endothelial TLR2 expression and a subsequent increase in NO bioavailability.

1. Introduction

Diabetes is one of the most prevalent chronic diseases in the United States, with approximately 9% of the population (29 million) suffering from the disorder. The vast majority (upwards of 95%) of these patients are type 2 diabetics [1], indicating a pressing need for the development of new and improved therapeutic options for treating this disease. Diabetes is well known to cause both macrovascular and microvascular complications, which can lead to the development of pathologies such as stroke and cognitive impairment [2,3]. The cerebrovasculature is comprised of large extracranial and intracranial arteries (e.g. the basilar artery), and these vessels contribute to the development of vascular resistance and regulation of cerebral blood flow [4,5]. It has been shown that a reduction in cerebral blood flow as a result of vascular dysfunction and damage can lead to the development of cognitive impairment [6]. While our current understanding of the complex mechanisms involving the development of vascular cognitive impairment in diabetes remains limited, there is a clear need for new therapeutic approaches to combat this process.

ET-1 is predominately generated by vascular endothelial cells, and acts through either the ETA receptor or ETB receptor to exert its effect on vascular function. The ETA receptor on smooth muscle cells is thought to be primarily responsible for the vasoconstrictive properties of ET-1, while the ETB receptor on endothelial cells leads to the release of nitric oxide and subsequent vasodilation [7–9]. In diabetes, circulating plasma levels of ET-1 are elevated in both human patients as well as in the Goto-Kakizaki (GK) rat, a lean type 2 diabetes animal model [10–12]. We reported that glycemic control with metformin [10] attenuated this increase in ET-1 levels in the GK rat. This was associated with improvement of cerebrovascular dysfunction characterized by hyperreactivity to ET-1, and impaired endothelial-dependent vasorelaxation [13,14]. However, mechanisms contributing to cerebrovascular dysfunction in diabetes are multifactorial and not fully understood. The role of inflammation in diabetes and its associated complications is an increasingly studied area. Recent studies have shown that Toll-like receptors (TLRs), in particular TLR2 and TLR4, are involved in the

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development of diabetic microvascular disease [15,16]. TLR2 has been implicated in vascular inflammation leading to decreased endothelial function [17,18] as well as playing a crucial role in the development of diabetic nephropathy [19]. However, the role of TLR2 in the development of diabetic cerebrovascular dysfunction and possible interaction with the ET system remains uncharacterized.

A relatively new treatment for diabetic patients are DPPIV inhibitors, which are oral hypoglycemic agents. DPPIV is a widely expressed peptidase that exists both in a transmembrane and a catalytically active soluble form [20]. Many substrates of DPPIV have been identified, the most well categorized of which are the incretins GLP-1 and GIP-1. DPPIV inhibitors such as linagliptin and sitagliptin prevent DPPIV induced cleavage of GLP-1 and GIP-1, which potentiates their insulin secreting and glucagon lowering effects. There has been recent evidence suggesting that inhibition of DPPIV may have beneficial pleotropic effects aside from their anti-hyperglycemic properties. Shah et al. [21] have reported inhibition of alogliptin modulates vascular tone independent of GLP-1 through nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) dependent pathways. Linagliptin, one of the more potent DPPIV inhibitors, has been shown to improve vascular function through anti-inflammatory and vasodilatory effects independent of its glucose-lowering properties [22]. This anti-inflammatory effect of DPPIV inhibitors was also demonstrated in mononuclear cells from type 2 diabetic patients treated with sitagliptin, where mRNA expression of TLR2 was reduced both after a single dose and twelve weeks of treatment [23]. However, the effect of DPPIV inhibition with linagliptin on ET-mediated cerebrovascular dysfunction and endothelial TLR2 expression has not been examined. In the current study, we hypothesized that linagliptin would restore cerebrovascular function through glucose dependent and independent effects via regulation of the ET-1 and TLR2 expression.

2. Methods

2.1. Animals

All experiments were performed using male Wistar rats from Harlan (Indianapolis, IN) and male diabetic GK rats (Taconic; Hudson, NY). The animals were housed at the Georgia Regents 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. Body weights and blood glucose measurements were taken biweekly. Blood glucose (BG) measurements were taken from tail vein samples using a commercially available glucometer (Freestyle, Abbott Diabetes Care, Inc.; Alameda, CA).

2.2. Animal treatments

Linagliptin treatment in both nondiabetic and diabetic rats was started at 24 weeks of age and was given for a period of 4 weeks. As other studies have reported, and on suggestion from the supplier of linagliptin (Boehringer Ingelheim Pharmaceuticals, Inc.), we initially began treating the rats with 83 mg of linagliptin/kg of chow. This treatment corresponds to plasma levels of ~100 nM and a 5 mg/kg oral dose. Upon monitoring of blood glucose levels for a period of one week, we did not notice any blood glucose reduction in the treated rats, and subsequently doubled the dose of linagliptin to 166 mg/kg of chow. Still no effect on blood glucose levels was noted, which was a similar to findings published by others [24,25]. Drug delivery was confirmed using a DPPIV activity assay that showed a reduction in plasma DPPIV activity levels in the treated groups. Inclusion in the diabetic groups was based on the presence of hyperglycemia, which was defined as fasting blood glucose levels above 180 mg/dL.

For the vascular function experiments in diabetic GK rats examining the impact of TLR2 antagonism on basilar artery function, male rats aged 12 weeks were utilized. We have previously shown that GK rats in this age range (10–12 weeks) exhibit cerebrovascular dysfunction characterized by hyperreactivity to ET-1 [13,14].

2.3. Endothelin-1 chemiluminescent immunoassay

At the end of the treatment duration, blood was collected via cardiac puncture under anesthesia with ketamine/xylazine (80 mg/kg:10 mg/kg IP). ET-1 levels in the plasma of both linagliptin treated and untreated Wistar and GK rats were assessed using the QuantiGlo Endothelin-1 ELISA kit (Bioteck, R&D, USA) according manufacturer's protocol and reported as total ET-1 levels.

2.4. Determination of vascular function

Animals were anesthetized and decapitated. The brain was quickly excised for isolation of basilar arteries, and vessels were cut in to 2 mm segments. Isometric tension exerted by the vessels was recorded via a force transducer using the wire-myograph technique (Model 610M, Danish Myo Technologies, Denmark). The myograph chambers were filled with Kreb's buffer (118.3 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.5 mM $CaCl_2$, and 11.1 mM dextrose), gassed with 95% O_2 and 5% CO_2, and maintained at 37 °C. Vessel segments were mounted in the chamber using 40-μm-thin wires and adjusted to a baseline tension (0.5 g). After stabilization, the vessels were challenged with 120 mM KCl, washed and allowed to equilibrate for 30 min, and endothelial integrity was assessed by pre-contraction with serotonin (1 μM) and subsequent addition of acetylcholine (ACh, 1 μM). Cumulative dose-response curves to 0.1–100 nM ET-1 were performed and the force generated was expressed as percent change from baseline. Maximum contractile response and area under the curve (used as an index of total contraction) were assessed, as well as sensitivity to ET-1 as measured by EC_{50}. In the experiments examining the impact of TLR2 antagonism on ET-1 mediated basilar contraction in diabetic GK rats, 30-min pre- incubation with antibody directed against the TLR2 receptor (Santa Cruz, 5 μg/mL) was used.

Endothelium-dependent relaxation to 1 nM–50 μM ACh was assessed after vessels were constricted to 60% of the baseline tension with 5-HT either alone or with a 30-min pre- incubation with antibody directed against the TLR2 receptor (Santa Cruz, 5 μg/mL). Sensitivity (median effective concentration [EC_{50}]) and total relaxation response (area under the curve) were calculated from the respective dose-response equations.

2.5. Cell culture

Brain microvascular endothelial cells (BMVECs) from 10 to 12 week old Wistar or GK rats were isolated as described previously [26,27]. Cells were grown in MCDB131 medium (Thermo Fisher, Waltham, MA, catalog: 10372-019). Cells were switched to serum-free medium 6 h before treatment with linagliptin 100 nM for 24 h. Experiments were performed using cells between passages 3 and 5.

2.6. Western blots

Cells were washed with PBS following treatment and harvested in ice cold Tris/HCl buffer (50 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM), and 2-mercaptoethanol (12 mM), phenylmethylsulfonyl fluoride (0.2 M), sodium orthovanadate (0.1 M), sodium fluoride (1 M), protease inhibitor cocktail (1:100, Sigma Aldrich, catalog: P8340), phosphate inhibitor cocktail 2 (1:100, Sigma Aldrich, catalog: P5726), and phosphatase inhibitor cocktail 3 (1:100, Sigma Aldrich, catalog: P0044). Samples were sonicated for 3 bursts of 10 s, and 4× LDS sample buffer was added. Equal protein loads (20 μg) of cellular lysate were boiled and separated on a 4–12% SDS-polyacrylamide gel by electrophoresis. Rat specific anti-TLR2 (1:1000, Abcam, Cambridge, MA, USA, catalog: ab108998) and