Type 2 diabetes is a strong risk factor for stroke. Linagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor in clinical use against type 2 diabetes. The aim of this study was to determine the potential antistroke efficacy of linagliptin in type 2 diabetic mice. To understand whether efficacy was mediated by glycemia regulation, a comparison with the sulfonlurea glimepiride was done. To determine whether linagliptin-mediated efficacy was dependent on a diabetic background, experiments in nondiabetic mice were performed. Type 2 diabetes was induced by feeding the mice a high-fat diet for 32 weeks. Mice were treated with linagliptin/glimepiride for 7 weeks. Stroke was induced at 4 weeks into the treatment by transient middle cerebral artery occlusion. Blood DPP-4 activity, glucagon-like peptide-1 (GLP-1) levels, glucose, body weight, and food intake were assessed throughout the experiments. Ischemic brain damage was measured by determining stroke volume and by stereologic quantifications of surviving neurons in the striatum/cortex. We show pronounced antistroke efficacy of linagliptin in type 2 diabetic and normal mice, whereas glimepiride proved efficacious against stroke in normal mice only. These results indicate a linagliptin-mediated neuroprotection that is glucose-independent and likely involves GLP-1. The findings may provide an impetus for the development of DPP-4 inhibitors for the prevention and treatment of stroke in diabetic patients. Diabetes 62:1289–1296, 2013

Type 2 diabetes is a strong risk factor for severe stroke. In addition, stroke patients with type 2 diabetes show higher stroke recurrence and mortality compared with nondiabetic stroke patients (1–4). Finally, a prediabetic state with impaired glucose tolerance is often detected in stroke patients after hospital admission, and such patients generally exhibit a poor prognosis (5,6).

Glucagon-like peptide-1 receptor (GLP-1R) agonists are novel treatments in clinical use against type 2 diabetes (7). They specifically bind G-protein–coupled GLP-1R, enhancing insulin secretion and decreasing glucagon production in a glucose-dependent manner (8). Besides its glucoregulatory action, the activation of GLP-1R by the specific ligand exendin-4 is efficacious against stroke in diabetic and nondiabetic animal models (9–13). In addition, GLP-1R activation by exendin-4 has proven beneficial in other animal models for neurodegenerative diseases such as Parkinson’s (14–16), Alzheimer’s (17–19), and Huntington’s (20). Finally, anti-inflammatory (15,21) and neurogenic (14,22,23) actions mediated by GLP-1R activation have been recently reported. Whether all effects of GLP-1 and its mimetics are mediated by the known GLP-1R is not yet completely clear because GLP-1R–independent activation pathways have only recently been reported (24).

In addition to GLP-1R agonists, GLP-1R activation can also be achieved through the prolongation of the short half-life of the endogenous GLP-1 by inhibition of the enzyme dipeptidyl peptidase-4 (DPP-4) (25). Upon food ingestion, intestinal endocrine L cells secrete GLP-1. However, GLP-1 is rapidly degraded by the enzyme DPP-4, which proteolytically removes two amino acids from the N-terminal end of GLP-1, thereby abolishing its interaction with GLP-1R. Thus, GLP-1 as such has no clinical use. This limitation has been overcome by the development of specific DPP-4 inhibitors (26). In addition to GLP-1, DPP-4 has many other substrates, including peptides with potential neurotrophic or neuroprotective effects (27).

Linagliptin is a recently approved DPP-4 inhibitor for the treatment of type 2 diabetes in monotherapy or combined with other antidiabetic drugs (28). Furthermore, some studies have suggested beneficial effects of linagliptin on secondary cardiovascular end points such as stroke (29,30). The aim of this study was to determine the potential efficacy of linagliptin against stroke in diabetic and normal mice by using a drug administration paradigm and a dose that mimics a type 2 diabetic and obese patient receiving DPP-4 inhibitor therapy. As a glycemic comparator, we used the sulfonlurea glimepiride.

RESEARCH DESIGN AND METHODS

Animals and experimental groups. The stroke experiments used 44 male C57Bl mice. In the first set of experiments, 21 8-week-old mice were exposed to a high-fat diet (HFD; Research Diets, Inc., New Brunswick, NJ) for 32 weeks (Fig. 1). Body weight was measured every fifth week. The intraperitoneal glucose tolerance test (IPGTT) and IP insulin tolerance test (IPinsTT) were carried out before and 12 weeks after the HFD treatment. When IPGTT and IPinsTT verified the animals’ diabetic state, the drug treatment was not started for an additional 13 weeks to mimic the clinical situation of an overtly diabetic patient who later suffers a stroke. We thus wanted to allow for metabolic toxicity of hyperglycemia and other diabetes manifestations to affect the body and the central nervous system.

Before the start of the linagliptin (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) and glimepiride (Sigma Aldrich, Stockholm, Sweden) treatments at week 25, baseline fasting blood glucose concentrations were measured and the animals assigned to the different treatment groups so that mean blood glucose values were equalized. The treatment groups thus created were tested for normality using the Shapiro-Wilk normality test.
Starting from week 25, all HFD-fed mice received oral administration of 10 mg/kg/body weight (bw) linagliptin daily (n = 7), 2 mg/kg/bw glimepiride daily (n = 7), or vehicle (n = 7) for 4 weeks before being subjected to stroke at week 29 (Fig. 1). The glimepiride and linagliptin treatments were continued 3 weeks until the animals were killed (Fig. 1).

In a second set of experiments, 23 10-week-old mice fed a normal diet were treated, as mice in the first experiment, for 4 weeks with 10 mg/kg/bw linagliptin daily (n = 7), 2 mg/kg/bw glimepiride daily (n = 7), or vehicle (n = 9). After 4 weeks of drug treatment, all mice were subjected to stroke, and the treatments were continued for an additional 3 weeks until they were killed.

All experiments were conducted according to the “Guide for the Care and Use of Laboratory Animals” published by U.S. National Institutes of Health (NIH publication #85–23, revised 1985) and approved by the regional ethics committee for animal experimentation.

**IPGTT and IPinsTT.** IPGTT and IPinsTT were carried out before the HFD treatment began and at week 12 (Fig. 1). The mice were fasted for 5 h, and intraperitoneal injections of 3 g/kg/bw glucose or 1 unit/kg/bw insulin were given. Blood was drawn from the tail vein, and glycemia was measured using a One-Touch Ultra 2 glucometer (LifeScan, Milpitas, CA) immediately before (time 0) and at 5, 10, 30, 60, and 120 min after the injection.

**Transient middle cerebral artery occlusion.** The intraluminal filament model of focal ischemia was used (31). All animals received linagliptin, glimepiride, or vehicle treatments 1 h before surgery. Anesthesia was induced by 3% isoflurane and continued during surgery with 1.5% isoflurane using a snout mask. Briefly, the carotid arteries on the left side were exposed, the external carotid was ligated, and temporary sutures were placed over the common carotid artery. Through a small incision in the external carotid artery, a 7-0 monofilament coated with silicone was advanced through the internal carotid artery until it blocked the origin of the middle cerebral artery. When the filament had been positioned, wounds were closed and anesthesia was discontinued. After 30 min of occlusion, the mice were anesthetized again, the filament was withdrawn, and the ligatures were removed from the common carotid artery. Body temperature was maintained between 36 and 38°C with a heat lamp during surgery and ischemia. The mice were transferred to a heated box where they regained wakefulness and were kept for 2 h. The surgeon performing the operation was blinded to the treatment groups.

**Measurements of fasting and fed blood glucose levels.** Fasting blood glucose levels were measured after 4 weeks of drug treatment. To do so, animals were given linagliptin, glimepiride, or vehicle and fasted for 5 h. Fed blood glucose levels were measured 1 h after drug treatment immediately before middle cerebral artery occlusion (MCAO), under anesthesia. Blood was drawn from the tail vein, and glycemia was measured using the One-Touch Ultra 2 glucometer.

**Measurements of DPP-4 activity and active GLP-1 levels.** To measure the DPP-4 enzymatic activity and levels of active GLP-1, all animals received linagliptin, glimepiride, or vehicle treatments, and blood was collected 1 h thereafter on the day they were killed. Plasma total active DPP-1 concentrations were determined by means of a GLP-1 assay kit (Meso Scale Discovery, Gaithersburg, MD). DPP-4 activity was detected, as recently published by our group (32).

**Brain immunocytochemistry.** Animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. The brains were extracted, postfixed in 4% paraformaldehyde overnight at 4°C, and submersed in 20% sucrose in phosphate buffer until they sank. A sliding microtome was used to cut 40-μm-thick coronal sections, which were stained as free-floating sections. The primary antibody anti-NeuN (1:100; Millipore Corp., Billerica, MA) was used to stain surviving neurons in striatum and cerebral cortex. Sections were incubated with the primary antibody for 36 h at 4°C in phosphate buffer containing 3% normal horse serum and 0.25% Triton-X. Primary antibody was detected using biotin-conjugated anti-mouse (Vector Laboratories, Burlingame, CA) secondary antibody (1:200). Sections were incubated with secondary antibody for 2 h at room temperature in phosphate buffer containing 3% normal horse serum and 0.25% Triton-X. For chromogen visualization, avidin-biotin complex (ABC kit, Vector Laboratories) and diaminobenzidine were used. For GLP-1R/NeuN staining, the sections were first microwave-boiled in citrate buffer for 10 min and then incubated with NeuN (1:100; Millipore) and GLP-1R (1:1,000; Abcam, Cambridge, U.K.) antibodies at 4°C overnight. The primary antibodies were visualized using Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibodies (1:200; Invitrogen, Paisley, U.K.) for 2 h at room temperature in phosphate buffer containing 3% normal donkey serum and 0.25% Triton-X. The sections were counterstained with DAPI.

**Brain infarct volume and cell quantifications.** An investigator blinded to the experimental groups performed tissue damage quantification and cell counting. For tissue damage evaluation, the NeuN-labeled tissue sections were displayed live on the computer monitor, and the area of contralateral hemisphere and the area of the intact ipsilateral tissue were measured in every section containing stroke damage using NewCast software (Visiopharm, Hoersholm, Denmark). To compensate for the stroke-induced morphologic tissue changes, the infarct volume was calculated by subtracting the volume of remaining intact tissue in the ipsilateral hemisphere from the volume of the contralateral hemisphere. The stroke volume in linagliptin and glimepiride groups has been normalized to its own respective vehicle-treated group (HFD and normal).

Immunoreactive cells were counted using a computerized unbiased setup for stereology, driven by NewCast software. The number of neurons was quantified using the optical fractionator method (33,34). Briefly, brain sections were displayed live on the computer monitor and the striatum and cortex were evaluated at low magnification. Quantifications were performed using an oil immersion lens (original magnification ×100) with a numeric aperture of 1.30. Ten evenly spaced sections in parallel-cut series through the entire striatum were included. Random sampling was carried out using the counting frame, which systematically was moved at predefined intervals so that ~300 immunoreactive cells were counted. The total number of cells was estimated according to the optical fractionator formula (33,34).

**Statistics.** Statistical analyses were performed using the Student unpaired t test or one-way ANOVA, followed by the Bonferroni post hoc test. Differences between groups were considered statistically significant when P < 0.05. Data are presented as means ± SEM.

**RESULTS**

**HFD exposure leads to insulin resistance, glucose intolerance, and hyperglycemia.** After 25 weeks of HFD treatment, the mice exhibited ~200% weight gain (Fig. 2A). As previously described (35), the HFD feeding led to insulin resistance, glucose intolerance, and hyperglycemia. The HFD-fed mice developed these metabolic derangements already after 12 weeks on this diet (Fig. 2B–D).

**Linagliptin inhibits DPP-4 activity, raises blood GLP-1 levels, and regulates glycermia.** Seven weeks of linagliptin treatment in HFD-fed mice, as well as in normal mice, significantly inhibited DPP-4 activity (Fig. 3A and E), leading to a 20- to 40-fold increase of blood GLP-1 levels (Fig. 3B and F), whereas glimepiride had no effect on these parameters. The results also show that linagliptin and glimepiride treatment decreased fed and fasting blood glucose levels in HFD-fed mice (Fig. 3C and D), while—as expected—in normal diet–fed mice, only glimepiride reduced glycermia (both fed and fasting; Fig. 3G and H).

**Linagliptin decreases ischemic brain damage.** To determine the potential antistroke efficacy mediated by linagliptin in HFD-treated mice, the brain infarct volume was assessed at 3 weeks after stroke. This measurement
revealed that linagliptin treatment showed a noticeable, albeit not statistically significant, trend toward reduction of ischemic tissue damage, whereas glimepiride did not (Fig. 4A). We previously show that stereological counting of surviving neurons in stroke-damaged striatum and cortex provides a highly accurate method, considerably more sensitive than merely estimating infarction volume, to quantify the antistroke efficacy of candidate drugs (10). Thus, to further and in greater detail assess the neuroprotective effect of linagliptin and glimepiride after stroke, NeuN-positive neurons were quantified in both stroke-damaged striatum and cortex using the optical fractionator method (see RESEARCH DESIGN AND METHODS). Consistent with the volume measurements, no increase of neuronal survival in the cortex and/or striatum was evident in glimepiride-treated mice (Fig. 4B–D). In contrast, in linagliptin-treated animals the cortex contained 30% more surviving neurons, a statistically significant effect above both vehicle and glimepiride (Fig. 4C). This effect remained statistically significant when data from the cortex and striatum were pooled (Fig. 4D).

To determine whether a diabetic background influences the linagliptin-mediated antistroke efficacy, the same type of experiment was performed in normal nondiabetic mice. The results also indicate that glimepiride showed a strong, albeit not statistically significant, trend toward reduction of ischemic tissue damage (Fig. 4E). When counting the total number of surviving NeuN-positive neurons in cortex and striatum, we observed a similar neuroprotective effect of linagliptin mainly limited to cerebral cortex (Fig. 3G and H), resembling the findings in diabetic conditions (Fig. 4C and D). In addition and contrarily to the findings in the diabetic mice (Fig. 4C and D), the results show that glimepiride induced a statistically significant neuroprotective effect, similar to that of linagliptin, in nondiabetic mice (Fig. 4G and H).

**GLP-1R is expressed in mouse brain neurons.** To determine GLP-1R expression in the brain, we performed immunohistochemical staining in the cortex/striatum of HFD-treated mice without stroke. Double staining with GLP-1R/NeuN revealed that GLP-1R was expressed exclusively in neurons, with the strongest expression levels in cortical pyramidal neurons. Virtually no cell that was negative for NeuN was positive for GLP-1R (Fig. 5).

**DISCUSSION**

Most preclinical studies that aim to prove the antistroke efficacy of candidate drugs are performed in experimental settings bearing little—if any—resemblance to clinical reality, which is a possible reason for several neuroprotective
drug failures (36,37). Potential causes of this lack of success include use of preclinical drug administration paradigms not achievable at the clinic level (e.g., drug administration before or very shortly after stroke, intracerebroventricular injections, and too-high doses of the candidate drugs (38)), efficacy experiments performed using animal models that lack common comorbidities of stroke patients, such as diabetes and hypertension (36), and finally, nearly all rodent stroke studies are performed in young animals, whereas most stroke patients are elderly (39).

Our goal in the current study was to determine the potential antistroke efficacy of a DPP-4 inhibitor therapy by mimicking the likely clinical scenario of an obese type 2 diabetic patient receiving this treatment suffering a stroke. To this end, we used middle-aged obese and diabetic mice and a drug administration route and dosages resembling a type 2 diabetic patient receiving chronic linagliptin treatment. Linagliptin is a recently approved DPP-4 inhibitor for the treatment of type 2 diabetes (28). Our results show a significant antistroke efficacy mediated by linagliptin treatment. To understand whether the neuroprotective efficacy by linagliptin was direct or rather secondary to its glycemic effects, we used two strategies: 1) We determined whether linagliptin showed antistroke efficacy also in nondiabetic mice, and 2) we performed a head-to-head comparison of linagliptin with the sulfonamide glimepiride, which does not affect the incretin system.

By comparing the linagliptin antistroke effects in type 2 diabetic versus normal mice, our results show that linagliptin is strongly efficacious against stroke in both phenotypes. The effect was even stronger in nondiabetic mice. They also point to an effect that occurs mainly in the ischemic penumbra (peri-infarct cortex). Our stroke model results in ischemic damage that originates in the striatum and then spreads across the overlaying cortex, depending on the duration of the MCAO. In contrast to the striatal damage, the cortical damage in our model is typically limited to a general decrease in neuronal density, often without clearly defined borders from the primary stroke-damaged areas. This prevents the accurate estimation of ischemic damage by only using volume measurements. On the contrary, stereologic quantifications of neurons can accurately identify the differences within such infarct areas, and therefore, is less likely to overlook the neuroprotective effects of a potential treatment. A typical change in neuronal density in the cortex after MCAO and the effect of drug treatments is illustrated in Fig. 4I–R. Thus, the cortex in our model contains mostly ischemic penumbra, where a neuroprotective intervention can be more effective, and that is exactly where we found most of the antistroke effect mediated by linagliptin.

Glimepiride treatment showed a stronger effect than linagliptin in decreasing glycemia in type 2 diabetic and nondiabetic mice. As expected in the latter, no changes in
FIG. 4. Neuroprotective effects of linagliptin and glimepiride treatments. A: Ischemic volume (mm$^3$) after 30 min of MCAO in HFD-fed mice. Number of surviving neurons in stroke-damaged striatum (B), cortex (C), and striatum and cortex combined (D) in HFD-fed mice. E: Ischemic volume (mm$^3$) after 30 min of MCAO in nondiabetic mice. Number of surviving neurons in stroke-damaged striatum (F), cortex (G), and striatum and cortex combined (H) in nondiabetic mice. The dashed lines in B, C, F, G, represent the average number of neurons in the brain areas of naïve animals (no stroke) where the neuronal quantification was performed. Bars represent means ± SEM. One-way ANOVA, followed by Bonferroni post hoc tests, was used. *$P < 0.05$, **$P < 0.01$. K: An illustration of typical brain damage in our stroke model. I and J: Photomicrographs of the area of the cortex illustrated in L on the contralateral, nondamaged, side of the brain, show normal neuronal density. Photomicrographs of the area (L) of the stroke-damaged cortex in HFD (M–O) and normal diet (P–R) illustrating the changes in neuronal density in vehicle, linagliptin, and glimepiride-treated mice, respectively. All photomicrographs have been enhanced with high-contrast monochromatic adjustment for better visual representation on small images.
blood glucose levels were observed after linagliptin treatment. Despite this, linagliptin treatment led to decreased DPP-4 activity, increased levels of blood GLP-1, and neuroprotection. Collectively, our results strongly suggest that the neuroprotective effect by linagliptin is unrelated to its glycemic actions. Because it mimicked the neuroprotective effects by the GLP-1R agonist exendin-4 previously reported by us and others (as noted earlier), this likely occurs by the increased GLP-1 levels observed. This is further supported by the fact that GLP-1 has been shown to pass the blood–brain barrier (40), whereas linagliptin does not (41). Our results also indicate the linagliptin-mediated neuroprotection occurs directly at the neuronal level because we found GLP-1R expression exclusively in neurons, with the strongest expression (based on immunohistochemistry) in cortical pyramidal neurons (Fig. 5). However, other peptides and substrates of DPP-4 (27,42) with reported neuroprotective and neurogenic actions, such as pituitary adenylate cyclase-activating polypeptide (43), glucose-dependent insulinotropic peptide (44), and stromal cell-derived factor 1α (45) may also be involved in the neuroprotective action mediated by linagliptin.

The results obtained by comparing linagliptin and glimepiride are intriguing because linagliptin was efficacious against stroke in both nondiabetic and diabetic mice, whereas glimepiride was efficacious only in nondiabetic mice. Because insulin has been suggested to have direct (nonglycemic) neuroprotective effects in the brain (46), we hypothesize that glimepiride-mediated efficacy against stroke in nondiabetic mice results from a direct neurotrophic effect mediated by increased insulin secretion and that this effect cannot be fully achieved in diabetic mice. Indeed, a nutritional regimen based on an HFD has been shown to render the brain insulin-resistant (47,48), thus potentially decreasing the neuroprotective actions mediated by insulin against stroke (46). In support of this hypothesis are also the inconclusive results from clinical trials aimed at assessing the role of tight glucose control against stroke in type 2 diabetes by using insulin as well as the sulfonylurea glimepiride (3,49). In line with our observed effects of linagliptin and glimepiride in diabetic animals are the recently observed results from a phase 3 trial in type 2 diabetic patients showing reduced incidences of stroke in linagliptin- versus glimepiride-treated patients (30).

The results obtained in our study have two implications of potential clinical relevance: First, they could be pertinent to type 2 diabetic patients receiving chronic linagliptin treatment. DPP-4 inhibition in these patients could decrease the risk of developing severe brain damage after a stroke while at the same time provide glycemic control without hypoglycemic side effects (6).

Second, given the glucose-independent effects of linagliptin, they advocate the use of DPP-4 inhibition as secondary prevention in nondiabetic and type 2 diabetic patients to minimize the damaging effects of recurrent stroke. Individuals who suffer a stroke or transient ischemic attack, in particular diabetic subjects, are at very high risk for another cardiovascular event (50). Thus, these patients could be prescribed DPP-4 inhibition therapy (safe and with minimal side effects) (28) aiming at reducing these types of complications. To test the feasibility of this hypothesis, we encourage preclinical and also clinical efforts in future work.

In conclusion, by using an experimental paradigm applicable to the clinical situation, we report the efficacy of linagliptin against stroke that is essentially glucose-independent and likely involves GLP-1. Furthermore, we demonstrate that linagliptin mediates neuroprotection in both type 2 diabetic and normal mice. Finally, we show significant differences between the linagliptin and glimepiride neuroprotective effects in normal versus diabetic background underlining the importance of performing this type of study in view of designing clinically suitable strategies. We believe that these findings provide an impetus for the further development of incretin-based drugs for the prevention and treatment of stroke in both diabetic and nondiabetic high-risk patients.

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V.D. designed and performed the stroke experiments, part of the immunohistochemistry studies, and stereochemistry analysis; acquired and processed images and figures; contributed to discussion; and wrote the manuscript. H.O. planned and performed bioactivity studies and contributed to discussion. A.O. performed NeuN/GLP-1R immunohistochemical staining and acquired and processed images. E.D. performed part of NeuN staining and quantification. P.W. performed bioactivity studies and drug administrations. T.N. provided expertise in GLP-1R detection and contributed to discussion. T.K. conceived the research plan, provided expertise in DPP-4 inhibitors and GLP-1R, coordinated GLP-1 and DPP-4 inhibition activity assays, contributed to discussion, and edited the manuscript. A.S. conceived the hypothesis and the research plan, provided expertise in diabetes and the HFD mice, contributed to discussion, and edited the manuscript. C.P. conceived, designed, and coordinated the research plan, contributed to discussion and to the stroke experiments, and wrote and edited the manuscript. V.D. and C.P. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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