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Linagliptin enhances neural stem cell proliferation after stroke in type 2 diabetic mice

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ABSTRACT

Dipeptidyl peptidase 4 (DPP-4) inhibitors are current drugs for the treatment of type 2 diabetes (T2D) based on their main property to enhance endogenous glucagon-like peptide-1 (GLP-1) levels, thus increasing insulin secretion. However, the mechanism of action of DPP-4 inhibition in extra pancreatic tissues has been poorly investigated and it might occur differently from that induced by GLP-1R agonists.

Increased adult neurogenesis by GLP-1R agonists has been suggested to play a role in functional recovery in animal models of brain disorders. We recently showed that the DPP-4 inhibitor linagliptin reduces brain damage after stroke in normal and type 2 diabetic (T2D) mice. The aim of this study was to determine whether linagliptin impacts stroke-induced neurogenesis.

T2D was induced by 25 weeks of high-fat diet. Linagliptin treatment was carried out for 7 weeks. Standard diet fed-mice were used as controls. Stroke was induced by middle cerebral artery occlusion 4 weeks into the linagliptin treatment. Neural stem cell (NSC) proliferation/neuroblast formation and striatal neurogenesis/gliogenesis were assessed 3 weeks after stroke. The effect of linagliptin on NSC viability was also determined *in vitro*.

The results show that linagliptin enhances NSC proliferation in T2D mice but not in normal mice. Linagliptin did not increase NSC number *in vitro* indicating that the effect of linagliptin on NSC proliferation in T2D is indirect. Neurogenesis and gliogenesis were not affected.

In conclusion, we found no correlation between acute neuroprotection (occurring in both T2D and normal mice) and increased NSC proliferation (occurring only in T2D mice). However, our results show that linagliptin evokes a differential response on NSC proliferation after stroke in normal and T2D mice suggesting that DPP-4 inhibition effect in the CNS might go beyond the well known increase of GLP-1.

1. Introduction

Stroke is a major cause of death and adult disability. Type 2 diabetes (T2D) strongly increases the risk of premature and severe stroke and stroke mortality [1,2]. Stroke patients with hyperglycemia at hospital admission generally show a poorer prognosis than non-diabetic individuals [1,3].

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from enteroendocrine L-cells following a meal [4–6]. GLP-1 enhances glucose-dependent insulin secretion *via* the specific G-protein-coupled GLP-1 receptor (GLP-1R) [7]. However, GLP-1 has a very short half-life mainly due to its rapid degradation by the proteolytic enzyme dipeptidyl peptidase 4 (DPP-4) [4]. The proteolytic removal of two single amino acids from the GLP-1*N*-terminus prevents GLP-1 from activating its receptor [8]. Consequently, GLP-1 as such is not a suitable drug candidate. To pharmacologically harness GLP-1 in the treatment of T2D and increase GLP-1 levels in the blood, stable GLP-1 analogues or mimetics and DPP-4 inhibitors have been developed [7,9–11]. Linagliptin is one of several DPP-4 inhibitors currently available in the clinic against T2D. Linagliptin is a potent and long-acting molecule [12,13] which is rapidly absorbed upon oral administration and highly

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selective for DPP-4, reducing its plasma activity by >80% over 24 h [13,14]. Unlike other DPP-4 inhibitors, linagliptin is mainly undergoing enterohepatic elimination, which is an advantage in T2D patients with nephropathy [15].

Neuroprotection conferred by GLP-1R agonists is established (see reviews [16–20]). GLP-1 and GLP-1R agonists have been shown to cross the blood–brain-barrier [21,22] and directly enhance neuronal survival since GLP-1R expression in the brain largely seems restricted to neurons [23–26]. On the other hand, indirect mechanisms may also contribute to the beneficial CNS effects of GLP-1R activation, including improved glycemia, reduced blood pressure, ameliorated endothelial dysfunction [27], and reduced brain inflammation [28,29]. Another potential mechanisms by which GLP-1R activation could produce beneficial effects in the CNS is through the regulation of neurogenesis [30]. Stroke induces neurogenesis in both rodents and humans. Adult neural stem cells (NSCs) in the subventricular zone (SVZ) generate neuroblasts that differentiate into neurons after migrating to the olfactory bulb [30]. Following stroke, a portion of these neuroblasts can migrate into striatum and replace dead neurons [31,32] suggesting that this process could play a role in functional recovery after stroke [33]. Interestingly, we and others have shown that GLP-1R agonists enhance adult neurogenesis in both normal and diabetic mice and rat models [34–39].

DPP-4 inhibition for the treatment of brain disorders is an emerging therapeutic concept. Recent studies have shown that the DPP-4 inhibitors sitagliptin and alogliptin exert neuroprotective effects in experimental Alzheimer and stroke models [40–42]. Recently, we showed that linagliptin is also neuroprotective in an animal model of stroke in T2D and in non-diabetic mice, independently of its glycemic effects [23]. Additionally, a human phase 3 study showed that patients with T2D had a reduced stroke incidence after linagliptin treatment [43]. Although DPP-4 inhibitors act via GLP-1 to stimulate insulin secretion, it is not known whether neuroprotection mediated by DPP-4 inhibitors occur through the same mechanism of action. In fact, DPP-4 inhibitors also modulate the activity of several other factors acting in CNS [44–47].

The potential effect of DPP-4 inhibition on adult neurogenesis has not been studied. The aim of this study was to determine the effect of the DPP-4 inhibitor linagliptin on NSC proliferation, neurogenesis and gliogenesis after stroke in both normal and diabetic mice.

2. Materials and methods

2.1. Animals, experimental design

Fourteen male C57Bl6/J mice (Nova SCB Stockholm, Sweden) were used in the *in vivo* experiments. High-fat diet (HFD) was given for 32 weeks until mice were sacrificed. At week 25 the linagliptin treatment was initiated and carried out for seven weeks till week 32. Standard diet fed-mice were used as controls. At week 29, stroke was induced by middle cerebral artery occlusion (see below). Linagliptin (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) was administered peri-orally by a gavage needle once daily at the dose of 10 mg/kg ($n = 7$) and vehicle ($n = 7$) was used as control.

To assess neurogenesis and gliogenesis, all animals were also given daily injections of the thymidine analog BrdU (50 mg/kg intraperitoneal) for two weeks following MCAO.

Brains were analyzed after sacrifice at week 32.

The experimental design, the model of HFD-mediated induction of a diabetic phenotype, the neuroprotetctive effect of linagliptin against stroke and its effects on glycemia have been recently described [23].

For the *in vitro* experiments with NSC, ten six-week old males C57Bl6/J (Nova SCB Stockholm, Sweden) were used.

All animal experiments were conducted under standardized housing conditions and according to the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH publication #85-23, revised 1985) and approved by the regional ethics committee for animal experimentation (S-713, S1710, S72-12).

2.2. Transient middle cerebral artery occlusion (tMCAO)

The intraluminal filament model of focal ischemia [48] was used to induce stroke 4 weeks into the linagliptin/vehicle treatment as recently described [23]. All animals received linagliptin or vehicle treatment 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 surgical incision in the external carotid artery, a 7–0 monofilament coated with silicone was advanced via the internal carotid artery until it blocked the origin of the middle cerebral artery. When the filament had been correctly positioned, wounds were closed and anesthesia was discontinued. After a 30 min ischemic occlusion time, the mice were re-anesthetized, the filament was withdrawn, and the ligatures were gently removed from the common carotid artery to regain blood flow. Body temperature was stabilized between 36 and 38 °C using a heating lamp and during anesthesia and ischemia. The mice were transferred to a heated box where they recovered under heat and supervision for 2 h. The surgeon was blinded to the treatment groups in these experiments.

2.3. Brain immunocytochemistry and cell quantifications

Animals were deeply anesthetized and transcardially perfused with 4% (*w*/*v*) paraformaldehyde. The brains were extracted and submersed in 20% sucrose in phosphate buffer overnight. 40-μm-thick coronal sections were cut throughout the brain using a sliding microtome and stained as free-floating sections. The following primary antibodies were used: mouse anti-Ki67 (1:200 dilution; Novocastra), a marker of cell proliferation; goat anti-DCX (doublecortin) (1:400 dilution; Santa Cruz Biotechnology), a marker for migrating neuroblasts; mouse anti-NeuN (1:100 dilution; Millipore), a neuronal marker; and rabbit anti-s100b (Millipore 1:100); a marker of astrocytes. A rat anti-BrdU (1:200 dilution; Santa Cruz Biotechnology) antibody in combination with anti-NeuN and anti-s100b was employed to assess neurogenesis and gliogenesis, respectively. Sections were incubated with primary antibodies overnight at 4 °C in a phosphate buffer containing 3% appropriate serum and 0.25% Triton X-100. Primary antibodies were detected by use of biotin- or Alexa 488- or Alexa 594-conjugated (Vector) secondary antibodies (1:200 dilution). Sections were incubated with secondary antibodies for 2 h at room temperature (approx. 21 °C) in phosphate buffer containing 3% of the appropriate serum and 0.25% Triton X-100. For chromogenic visualization, biotinylated secondary antibodies, avidin–biotin complex (ABC kit; Vector) and diaminobenzidine were used.

The cells were counted using the bright field or the epi-fluorescent microscope. Three consecutive brain sections spaced at 320 μm containing striatum (from Bregma 1 to 0.5 mm) were used for cell counting. The first section was chosen based on an anatomical location along the rostra-caudal axis (≈1 mm from Bregma). The second and the third sections were 320 and 640 μm caudally from the first section respectively.

2.3.1. Cell cultures and adult neural stem cell viability and proliferation assays

The SVZ of the lateral brain ventricles from five mice was microdissected and enzymatically dissociated and the cells were isolated and expanded as described by Mansouri et al. [49]. Neurospheres were split every five days for four weeks and all experiments were performed between passages 2 and 5.

To measure adult neural stem cell (NSC) viability, NSC were seeded as single cells into 96-well plates (Corning B.V. Life Sciences, Amsterdam, Netherlands) at the final concentration of 50,000 cells/well in Dulbecco's modified Eagle's medium/F12 supplemented with B27 and in 10 μg/L of epidermal growth factor (EGF). Under these conditions NSC viability