Original Article
DPP-4 inhibitor saxagliptin ameliorates oxygen deprivation/reoxygenation-induced brain endothelial injury

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Abstract: Cardiovascular diseases are the main cause of death and disability among diabetes patients. Atherosclerosis-associated stroke is one of the most severe complications in diabetes patients. DPP-4 inhibitors are a class of potent anti-glycemic agents used to treat diabetes. Recently, some DPP-4 inhibitors have been shown to have cardiovascular benefits. In this study, we reveal that saxagliptin, one of the most widely used DPP-4 inhibitors, exhibits vascular protective effects against oxygen and glucose depletion/reoxygenation (OGD/R) in human brain vascular endothelial cells. Our data show that DPP-4 is fairly expressed in brain endothelial cells and its expression is induced by OGD/R. The results of MTT assay show that inhibition of DPP-4 by saxagliptin ameliorates OGD/R-induced reduced cell viability, and LDH assay demonstrated that saxagliptin reduces cellular toxicity. Furthermore, we show that saxagliptin mitigates OGD/R-induced collapse of mitochondrial membrane potential (MMP). Saxagliptin also reduces oxidative stress-induced release of 4-HNE and the NAPDH oxidase catalytic subunit NOX-4. At the molecular level, saxagliptin suppresses OGD/R-induced expression of pro-inflammatory cytokines and production of vascular adhesion molecules including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, monocyte chemoattractant protein 1 (MCP-1), vascular cellular adhesion molecule 1 (VCAM-1), and E-selectin. Mechanistically, saxagliptin inhibits activation of the NF-κB pathway by OGD/R via its inhibitory effect on nuclear p65 and NF-κB promoter activity. Collectively, our study explicitly demonstrates the cellular protective effect of saxagliptin against OGD/R-induced brain endothelial injury. Our findings extend our recognition of the protective roles of DPP-4 inhibitors in brain vascular cells.

Keywords: Oxygen-glucose deprivation/reoxygenation (OGD/R), DPP-4, saxagliptin, vascular protection, NF-κB

Introduction

The increasing prevalence of diabetes in recent decades has continued to be a major threat to human health [1]. Chronic prediabetes and diabetic condition often cause a series of complications including renal, vision, neurological and vascular complications. Among these, cardiovascular diseases remain the principal cause of death and disability among patients with diabetes [2]. Diabetes causes various microvascular and macrovascular changes, which often culminate in major clinical complications such as stroke, heart failure etc. A meta-analysis of 102 diabetes risk studies shows that the hazard ratio for diabetes is 2.27 for ischemic stroke, the most frequent stroke type [3]. In diabetic patients, stroke often has poor prognosis due to their diabetic condition altering the metabolism, which causes multiple comorbidities and makes it difficult to cure the condition [4]. In diabetes patients, stroke often contributes to the disease-associated atherosclerotic burden. Individuals with diabetes are especially susceptible to complications of cerebral small vessel diseases [5]. The diabetic condition causes massive macrovascular and microvascular complications, which are closely associated with endothelial dysfunction. Endothelial dysfunction is often present in prediabetes patients, so targeting endothelial dysfunction is a desirable strategy for preventing vascular complications in diabetes patients [6].

The endothelium forms the internal layer of vessel walls and is the largest endocrine gland in
the human body [7]. The elaboration of a fine vasculature network ensures adequate supply of oxygen and nutrients, and maintains whole-body homeostasis. Endothelial dysfunction has been recognized as being critical factor in vascular complications and stroke in diabetes [6]. During brain vascular complication and ischemic stroke, interruption of cerebral blood flow dramatically reduces the availability of oxygen and glucose to brain cells, thereby impairing cellular ATP production and causing necrosis of brain tissues. To mimic the pathophysiological features of brain vascular injury and stroke, oxygen-glucose deprivation and reoxygenation (OGD/R) are often used to generate in vitro ischemic models in primary cultured endothelial cells [8].

Gliptins are a class of glucose-lowering agents used to treat type 2 diabetes [9]. More than a dozen gliptins have been developed for the treatment of T2DM, including the most commonly used alogliptin, linagliptin, saxagliptin, sitagliptin, and vildagliptin. Recently, several gliptins have been reported to have a beneficial effect on cardiovascular proteins [10, 11]. Saxagliptin, originally developed by Bristol-Myers Squibb, showed cardiovascular benefit in an experimental myocardial infarction animal model [12]. In our study, we aimed to examine the effects of the DPP-4 inhibitor saxagliptin in cultured brain endothelial cells in the context of oxygen-glucose deprivation/reoxygination.

Materials and methods

Cell culture and OGD/R treatment

Human subject researches were designed following the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Human subject experiments were approved by the ethics committee of Zhengzhou University (No. 201600035). Primary human brain microvascular endothelial cells (HBMVEs) were obtained from Cell Systems (ACBRI 376). Low passage (3-7) HBMVEs were maintained in Endothelial Cell Growth Medium-2 BulletKit (CC-3156) EBM-2 Basal Medium (CC-3162, Lonza) and supplemented with 10% fetal bovine serum (FBS). HBMVEs were pre-incubated with 500 nM or 1 μM saxagliptin (Bristol-Myers Squibb, USA) for 12 h. To deplete oxygen and glucose, HBMVEs were exposed to deoxygenated media in a sealed incubator chamber flushed with 1% O₂, 5% CO₂, and 94% nitrogen, then stored at 37°C for 8 h, followed by three washes with PBS and 24 h reoxygenation with normal culture media under normoxic conditions (21% O₂, 5% CO₂) at 37°C in a tissue culture incubator.

Real-time PCR analysis

Total RNA from cells was extracted using a High Pure RNA Kit from Roche. RNA concentrations were quantified by Nanadrop. A total of 1 μg RNA was used to synthesize cDNA using iScript Supermix from Invitrogen. SYBR-based real-time PCR experiments were performed to detect mRNA transcripts of human DPP-4, TNF-α, IL-6, MCP-1, VCAM-1, E-selectin and GAPDH on an ABI 7500 platform with a commercial kit (Thermo Fisher Scientific, USA).

Western blot analysis

After indicated treatment, HBMVEs were lysed using cell lysis buffer supplemented with protease inhibitor cocktails. Protein concentrations were measured using a commercial bicinechonic acid (BCA) kit (Thermo Fisher Scientific, USA). The total cell lysates (20 μg) were loaded onto a PAGE gel to separate the proteins by size. The separated protein mix was transferred onto PVDF membranes (Bio-Rad, USA). The PVDF membranes were blocked with 5% non-fat milk at RT for 1 h and incubated with primary antibodies overnight in a cold room. After 3 washes, membranes were then incubated with HRP-conjugated secondary antibodies for 2 h at RT. After washing 3 times with TBST, blots were visualized using an enhanced chemiluminescence reagent kit (Thermo Fisher Scientific, USA). β-actin was used as an internal control.

Nuclear extracts

Nuclear extracts of HMVECs were extracted using a kit from Thermo Fisher Scientific in accordance with the manufacturer’s instructions. The nuclear protein lamin B was used as a quality control, and the nuclear fraction of p65 protein level was examined to determine NF-κB activation.

ELISA

To measure the secreted levels of TNF-α, IL-6, MCP-1 and HMGB-1 in the different conditions, the HBMVEs culture media was collected for the analysis. Individual ELISA kits were pur-
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chased R&D Systems. The experiments were performed in accordance with the manufacturer’s instructions. The data were collected by 96-plate reader spectrometry. Absolute values were obtained using a standardized 4-PL curve. The relative level of each protein is presented as normalized to total protein amounts in each condition.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT is used to assay cell viability. Briefly, cells were incubated for 4 h with 0.8 mg/ml MTT in serum-free medium, followed by the addition of DMSO. The stabilized cell-MTT reaction mixture was transferred into 96-well plates and absorbance was recorded at 560 nm using a microplate spectrophotometer system.

Lactate dehydrogenase (LDH) cell toxicity experiment

LDH cytotoxicity was assessed based on leakage of LDH into the culture medium. The culture medium was determined to obtain cell-free supernatants from the different conditions. The activity of LDH in the mediums was determined using a commercially available kit from Sigma-Aldrich. The data were analyzed and are presented as percentages of the control values.

Mitochondrial membrane potential (MMP) assay

HBMVEs were pre-incubated with 0.5 and 1 μM saxagliptin for 12 h, followed by exposure to OGD/R. Cells were then washed 3 times and loaded with 20 nmol/L TMRM (Molecular Probe, USA) in phenol red free medium for 30 min. Fluorescent signals were visualized in the IBE2000 inverted fluorescence microscope (Zeiss, Germany). The fluorescence density of MMP was quantified with the software Image J. We defined the regions of interest (ROI) and counted the average number of cells present in the defined ROI. The integrated density value (IDV) of fluorescent intensity in ROI was evaluated. Average level of intracellular MMP=IDV/the average number of cells.

Reactive oxygen species (ROS) determination

HBMVEs were pre-incubated with 0.5 and 1 μM saxagliptin for 12 h, followed by exposure to OGD/R. Cells were then washed 3 times and loaded with 5 μM DCFH-DA in Hank’s balanced salt solution (HBSS) for 30 minutes. Fluorescence signals were recorded using the IBE-2000 inverted fluorescence microscope (Zeiss, Germany) with excitation at 510 nm and emission at 580 nm. The fluorescence density of MMP was quantified with the software Image J. We defined the regions of interest (ROI) and

Figure 1. Oxygen-glucose deprivation/reoxygenation (OGD/R) induces DPP-4 expression in primary human brain microvascular endothelial cells (HB-MVEs). HBMVEs were pre-incubated with 0.5 and 1 μM saxagliptin for 12 h, followed by exposure to OGD/R. A. Expression of DPP-4 at the mRNA level was determined by real-time PCR analysis; B. Expression of DPP-4 at the protein level was determined by western blot analysis (n=5).

Figure 2. Saxagliptin ameliorates OGD/R-induced reduced cell viability and lactate dehydrogenase (LDH) release in HBMVEs. HBMVEs were pre-incubated with 0.5 and 1 μM saxagliptin for 12 h, followed by exposure to OGD/R. A. Cell viability was determined by MTT assay; B. LDH release was determined (n=5-6).
counted the average number of cells present in the defined ROI. The integrated density value (IDV) of fluorescent intensity in ROI was evaluated. Average level of intracellular ROS=IDV/ the average number of cells.

**Promoter assay**

Cells were co-transfected with NF-κB promoter and a firefly luciferase promoter by Lipofectamine 2000 reagent from Invitrogen. Cells were pre-incubated with 0.5 and 1 μM saxagliptin for 12 h, followed by exposure to OGD/R. Release of HMGB-1 was measured by ELISA (*, #, $, P<0.01 vs. previous column group, n=5-6).

**Statistical analysis**

The SPSS statistical software package was applied for all data significance tests in this study. The paired samples were subjected to the student’s t-test. For data collected from more than 2 groups, statistical significance was tested using the ANOVA test.

**Results**

**OGD/R induces DPP-4 expression in human brain microvascular endothelial cells**

To examine the role of DPP-4 and its involvement in brain vascular function, we applied OGD/R and compared DPP-4 expression changes in human brain microvascular endothelial cells (HBMVEs). Interestingly, DPP-4 mRNA expression in HBMVEs was roughly 3 times higher than in the vehicle control after OGD/R (Figure 1A). We confirmed this OGD/R-associated elevation at the protein level. DPP-4 protein was close to 3-fold higher after OGD/R than in the vehicle (Figure 1B).

**Saxagliptin ameliorates OGD/R-induced reduced brain endothelial cell viability**

OGD/R is known to be a negative factor in brain endothelial cell survival. Based on the induction of DPP-4 by OGD/R, we explored the possible influence of inactivation of DPP-4 on HBMVEs by its antagonist, saxagliptin. We chose to use saxagliptin because it’s a widely used clinically available glycemic reducer. Firstly, we assessed the overall viability of brain endothelial cells upon the combined presence of OGD/R and saxagliptin via MTT assay. When the different experiment groups were compared to the non-treated cells, OGD/R caused a reduction in HBMVEs viability by about half, while blockade of DPP-4 by the addition of saxagliptin dramatically improved cell viability. The same OGD/R exposure caused only 30% and 15% cell death when HBMVEs were pre-incubated with 0.5 and 1 μM saxagliptin for 12 h, respectively (Figure 2A). Secondly, we assessed cellular toxicity by measuring LDH release. Compared to treated cells, non-treated cells had a basal LDH level of only 5%, while OGD/R caused LDH release of approximately 30%. The same exposure to OGD/R caused only roughly 18% and 10% LDH release when cells were pre-incubated with 0.5 and 1 μM of saxagliptin, respectively (Figure 2B). Therefore, our experiments demonstrate that blockage of DPP-4 by saxagliptin ameliorated OGD/R-induced cell death in brain endothelial cells.

**Saxagliptin inhibits OGD/R-induced HMGB-1 release**

High mobility group box 1 protein (HMGB-1) is a conserved stress responsive nuclear protein that is released into the extracellular space by endothelial cells. We measured the levels of endothelial-derived HMGB-1 in another treatment experiment. Compared to control cells, exposure of treatment group cells to OGD/R resulted in approximately 3-fold greater release of HMGB-1. However, the same condition of

![Image](https://via.placeholder.com/150)
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OGD/R exposure caused only roughly 2- and 1.5-fold greater release of HMGB-1 when the cells were pre-incubated with the two doses of saxagliptin, respectively (Figure 3).

Saxagliptin ameliorates OGD/R-induced mitochondrial dysfunction

OGD/R treatment is known to induce cellular mitochondrial dysfunction. We elected to assess whether saxagliptin-mediated DPP-4 inhibition could ameliorate mitochondrial dysfunction upon OGD/R in HBMVEs. We measured changes in mitochondrial membrane potential (MMP) under the different conditions by TMRM staining. Compared to non-treated cells, cells in the OGD/R treatment group saw a 55% reduction in MMP. However, the same exposure to OGD/R resulted in approximate 35% and 25% reductions in MMP in the presence of 0.5 and 1 μM saxagliptin (Figure 4).

Saxagliptin mitigates OGD/R-induced oxidative stress

Our experiments reveal the cellular protective effect of Saxagliptin against OGD/R-induced brain endothelial damage. Next, we assessed the mechanism through which saxagliptin exerts its protective effects under these circumstances. Since OGD/R has a profound influence as an inducer of oxidative stress, we measured the level of the oxidative stress byproduct 4-HNE and expression of NAPDH oxidase NOX-4. Compared to non-treated cells, exposure of the treatment group to OGD/R resulted in approximately 3-fold higher cellular 4-HNE, but only roughly 2- and 1.5-fold higher 4-HNE when HBMVEs were pre-incubated with the two doses of saxagliptin, respectively (Figure 5A). For NADPH oxidase, we assessed expression of NOX-4, the dominant catalytic subunit in the endothelium. Compared to non-treated cells, OGD/R resulted in approximately 3-fold NOX-4 elevation, while the same exposure to OGD/R resulted in only roughly 2.2- and 1.5-fold NOX-4 expression when HBMVEs were pre-incubated with the two doses of saxagliptin, respectively (Figure 5B). These findings confirm that treatment with saxagliptin offers protection against OGD/R-induced oxidative stress in HBMVEs.

Saxagliptin inhibits OGD/R-induced cytokine induction

OGD/R activates inflammatory pathways and results in production of pro-inflammatory cytokines. We examined the effect of saxagliptin on cytokine production in HBMVECs. We collected the cell culture media and measured the levels of three major cytokines: TNF-α, IL-6 and MCP-1. Changes in their mRNA expression are shown in Figure 6A. Compared to the control, OGD/R caused a significant increase in the expression of all three genes. However, pre-incubation with 0.5 and 1 μM saxagliptin significantly suppressed OGD/R-induced expression of TNF-α, IL-6 and MCP-1. We were also able to confirm inhibition of these factors at the protein level, as shown in Figure 6B. Compared to the control group, OGD/R caused approximately 3-fold greater release of these three cytokines. However, pre-incubation with the two doses of saxagliptin significantly inhibited OGD/R-induced release of TNF-α, IL-6 and MCP-1.

Saxagliptin inhibits OGD/R-induced expression of the vascular adhesion molecules VCAM-1 and E-selectin

OGD/R-induced endothelial damage can lead to the activation and release of vascular adhesion molecules. Next, we assessed the effect of saxagliptin on expression of two key vascular
adhesion molecules: VCAM-1 and E-selectin. At the mRNA level and compared to the control, OGD/R resulted in roughly 6-fold higher VCAM-1 mRNA transcripts, however VCAM-1 mRNA was only increased by roughly 3.5- and 2.2-fold when HBMVEs were pre-incubated with the two doses of saxagliptin, respectively (Figure 7A). Similarly, OGD/R caused approximately 7.5-fold higher E-selectin mRNA induction, compared to roughly 4.2- and 3-fold higher E-selectin mRNA when cells were pre-incubated with the two doses of saxagliptin, respectively (Figure 7A). We confirmed these inhibitions at the protein level. Under basal conditions, expression of both VCAM-1 and E-selectin protein is extremely low, while OGD/R induced expression of large quantities of these molecules. However, this effect was considerably weakened upon pre-incubation with the two doses of saxagliptin (Figure 7B).

Finally, we explored the molecular pathways involved in the protective effects of saxagliptin. We tested endothelial NF-κB as it is one of the major regulators of the production of the cytokines and vascular molecules measured in this study. We assessed accumulation of nuclear NF-κB p65 protein and transfection NF-κB promoter activity under the different conditions. To
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measure the level of nuclear p65, we extracted nuclear protein and compared the level of p65 to that of non-treated cells. Cells exposed to OGD/R had roughly 3-fold higher accumulation of nuclear p65, but only roughly 2.3- and 1.5-fold higher nuclear p65 when cells were pre-incubated with the two doses of saxagliptin, respectively (Figure 8A). When NF-κB promoter was transfected into the cells, OGD/R resulted in approximately 35-fold NF-κB promoter activation. However, OGD/R caused only roughly 20- and 5-fold promoter activation when the cells were pre-incubated with the two doses of saxagliptin, respectively (Figure 8B). The results of these experiments confirm the inhibitory effect of saxagliptin on OGD/R-induced activation of the endothelial NF-κB pathway.

Discussion

Brain function is dependent on its fine vasculature and circulation. The brain endothelium maintains oxygen transport and nutrient supply by forming the blood brain barrier. In the human brain, glucose is the obligatory source of energy for the brain [13]. Stroke occurs when the blood supply to brain is interrupted or reduced, and the supply of oxygen and glucose cannot meet the needs of brain cells. Oxygen and glucose deprivation resulting from ischemic stroke activates proteases, resulting in degradation of endothelial tight junction proteins and permeability changes in the blood brain barrier. Glucose deprivation in brain endothelial cells can lead to disruption of the function of the blood brain barrier, which leads to activation of immune cells which penetrate the endothelium and infiltrate into the brain tissue, initiating an inflammatory cascade that leads to neuronal damage and cell death [14].

In our in vitro cell culture model, the brain microvascular endothelial cells are exposed to OGD/R which mimic the ischemic condition of stroke. OGD/R exposure is detrimental to healthy HBMVEs, but pre-incubation with the DPP-4 inhibitor saxagliptin ameliorates much of the damage to brain endothelial cells resulting from OGD/R. Our study began by determining DPP-4 expression with or without OGD/R exposure. Our examination of DPP-4 expression in brain endothelial cells confirmed both mRNA and protein expression of DPP-4 in HBMVEs. Furthermore, our data show DPP-4 expression is inducible upon exposure to OGD/R at both the mRNA and protein levels. These facts lay the ground work for our hypothesis that brain endothelial DPP-4 is a responsive factor of OGD/R. Next, we explored every aspect of cellular function upon DPP-4 inhibition in the context of OGD/R. Our studies elucidate four facets of cellular protection by saxagliptin. Firstly, saxagliptin relieves OGD/R-induced cell death as revealed by MTT assay and assay of cytotoxicity assay via cellular release of LDH. Secondly, saxagliptin ameliorates OGD/R-induced cellular oxidative stress. Our data show that saxagliptin suppresses the release of 4-HNE and expression of the NAPDH oxidase catalytic unit NOX-4 triggered by OGD/R. 4-HNE is the secondary product of fatty acid oxidation and has been shown to be cytotoxic and genotoxic in brain endothelial cells [15]. Endothelial NOX-4 is the dominant catalytic unit of NAPDH oxidase in endothelial cells, which attributes to a great extent to ROS generation in endothelial cells [16]. The suppression of 4-HNE release and NAPDH oxidase activity.
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A pharmacokinetic study showed that saxagliptin has 5-fold higher DPP-4 inhibitive activity than vildagliptin, which may be due to its unique cyanopyrrolidine group on its chemical structure [21]. Preclinical studies show that administration of saxagliptin improves endothelial nitric oxide release and reduces vascular oxidative stress in a rat model [22, 23]. Diabetic rats treated with saxagliptin showed improved exercise adaption and vascular mitochondrial function including increased eNOS expression [24]. A clinical study showed that saxagliptin improves the function of circulating angiogenic cells and induces angiogenesis in type 2 diabetes patients [25]. Another clinical study showed that saxagliptin has a beneficial effect equivalent to that of metformin on circulating endothelial progenitor cells and endothelial function in newly diagnosed type 2 diabetic patients [26]. The most recent clinical trial suggests that a combined regime with saxagliptin and metformin increases circulating CD31-positive endothelial progenitor cells and improves the cardiovascular index, thereby suggesting that the combination of these two anti-diabetic agents provides a better therapeutic effect in early diagnosed diabetes before the development of macrovascular complications [27]. All these studies demonstrate that saxagliptin has a definite influence on vascular cell function.

In conclusion, our study extends the beneficial implication of saxagliptin to brain vascular endothelial cells. We explicitly show that saxagliptin has a direct positive influence on the survival and function of brain endothelial cells upon exposure to hypoxia and nutrient depletion, indicating that saxagliptin may have a beneficial function in damaged blood barrier function such as in stroke and brain injury. An excit-
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ing prospect would be for our next experiment to establish an animal stroke model to evaluate this protective effect of saxagliptin in vivo.

Disclosure of conflict of interest

None.

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