Alogliptin, a dipeptidylpeptidase-4 inhibitor, for patients with diabetes mellitus type 2, induces tolerance to focal cerebral ischemia in non-diabetic, normal mice

Dong Yang, Yukako Nakajo, Koji Iihara, Hiroharu Kataoka, Hiroji Yanamoto

Laboratory of Neurology and Neurosurgery, National Cerebral and Cardiovascular Research Center, Suita 565-8565, Japan
Department of Neurosurgery, National Cerebral and Cardiovascular Research Center, Suita 565-8565, Japan
Research Laboratory, Rakuwa-kai Otowa Hospital, Kyoto 607-8062, Japan
Department of Neurosurgery, China–Japan Friendship Hospital, Beijing, PR China
Department of Cardiovascular Science, Division of Surgical Medicine, Osaka University Graduate School of Medicine, Suita 565-0871, Japan

ABSTRACT

Effective interventions that provide obvious neuroprotection are currently fairly limited. Glucagon-like peptide-1 (GLP-1), an enhancer of insulin production with a trophic effect on β cells in the islets, has been found to be trophic for neuronal cells. Alogliptin benzoate (AGL), a selective inhibitor of dipeptidylpeptidase-4 (DPP-4) functioning as a long-acting agonist of GLP-1, is in clinical use worldwide for patients with diabetes mellitus type 2. To clarify whether administration of AGL, independent of the insulinotropic effect, protects the brain against focal ischemia, we investigated the effect of AGL on the development of cerebral infarction in non-diabetic normal mice. Male C57BL/6J mice were administered AGL (7.5, 15, or 30 μg) once a day for three weeks by intragastric gavage. After the induction of temporary focal ischemia, volumes of infarcted lesions and neurological deficits were analyzed at 24 h (acute phase) and seven days (chronic phase). In the acute phase, significant reductions were observed in the volumes of infarcted lesions (p = 0.009), and in the severity of neurological deficits (p = 0.004), in the group treated with 15 μg of alogliptin benzoate, but not the 7.5 or 30 μg-treated groups. This significant reduction in volumes of infarcted lesions persisted into the chronic phase. At the end of the AGL treatment; before the induction of ischemia, the levels of brain-derived neurotrophic factor (BDNF), a potent neuroprotectant in the brain, were elevated in the cortex (p = 0.008), or in the whole forebrain (p = 0.023). AGL could be used as a daily neuroprotectant or an enhancer of BDNF production aiming to attenuate cerebral injuries, for the growing number of people who have the risk of ischemic stroke.

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Corresponding author at: Laboratory of Neurology and Neurosurgery, National Cerebral and Cardiovascular Research Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. Fax: +81 6 6835 6894.
E-mail address: hyanamot@res.ncvc.go.jp (H. Yanamoto).
The first two authors contributed equally to this work.

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1. Introduction

For the growing number of people who have the risk of, or experienced cerebral infarction or TIA (Weimar et al., 2010; Hata et al., 2005), development of a novel compound to protect neurons from focal ischemia, or even to promote cerebral repair, is urgently required. In the incretin family, glucagon-like peptide-1 (GLP-1), or insulinotropic secreted from L cells in the gastrointestinal tract as a response to food ingestion (Cefalu, 2010; Rizzo et al., 2009), acts as a trophic factor for β cells in the islets by enhancing insulin biosynthesis/release and their proliferation (Turton et al., 1996). In addition to the β cell-trophic/insulinotropic effect, GLP-1 exerts a neurotrophic effect in the brain (McClean et al., 2010; Perry et al., 2002). Indeed, GLP-1 can enter the brain; the GLP-1 receptors (GLP-1R) is expressed widely in the central nervous system (During et al., 2003; Turton et al., 1996); and the activation of GLP-1R was found to improve cognitive performance (Li et al., 2010a; During et al., 2003). However, once secreted into the blood, GLP-1 is rapidly degraded and inactivated following release of the intrinsic antagonizing enzyme, dipeptidylpeptidase-4 (DPP-4).

Exendin-4 (Ex-4), a long-acting analog of GLP-1 (a GLP-R agonist), developed for intravenous treatment of type 2 diabetes mellitus (DM-2), demonstrated a neuroprotective property in vivo after cerebro-ventricular administration (Li et al., 2009). Ex-4 also exerted a neurotrophic property in vitro (Li et al., 2010c). Moreover, in a transgenic mouse model of Alzheimer’s disease (AD) combined with streptozocin-induced DM-2, a continuous subcutaneous injection of Ex-4 reduced the levels of amyloid-β (Aβ) protein in the brain (Li et al., 2010b).

Alogliptin benzoate (AGL), a potent and highly selective inhibitor of DPP-4, developed for once-daily oral treatment of DM-2, demonstrated a lower incidence of unfavorable side effects such as hypoglycemia and hyperphagia, compared to previous drugs (Moritoh et al., 2008; Feng et al., 2007). Although treatment with AGL for a prolonged period in DM-2 patients is expected to protect β cells and prevent atherosclerotic vascular damage, it is unknown whether AGL, independent of its insulinotropic properties, protects neurons against lethal ischemia. To clarify whether AGL acts as a neuroprotectant, we studied the effect of AGL given per os every day for three weeks prior to the insult, or once following the insult, on the development of cerebral infarction after the induction of ischemic stroke in non-diabetic normal mice.

Treatment with a DPP-4 inhibitor, vildagliptin improved the expression of genes and proteins responsible for insulin secretion, indicating that DPP-inhibitors may affect glucose metabolism-related gene and protein expression (Akarte et al., 2012). To clarify whether brain-derived neurotrophic factor (BDNF) levels are affected by AGL, we also studied alterations in BDNF levels in the brain after chronic prophylactic treatment with AGL.

BDNF, the most abundant neurotrophin in the brain, stimulates neural migration; promote neuronal differentiation; induce neurite outgrowth; enhance synapse formation, learning and memory, and neuronal survival; lower blood glucose levels; improve glucose/lipid metabolism, and reduce appetite and body weight (Yamamoto et al., 2000b, 2004; Nakagawa et al., 2003; Hofer and Barde, 1988). Increase in intracerebral BDNF levels, prior to the insult, induces tolerance to focal cerebral ischemia, and improve the functional outcome in rodent models of ischemic stroke (Nakajo et al., 2008; Galloway et al., 2008; Yamamoto et al., 2000a, 2000b, 2004, 2008). In contrast, a genetic decrease in BDNF levels in the brain increased volumes of infarcted lesions and worsened learning and memory (Yamamoto et al., 2011). Interestingly, BDNF levels in the brain were decreased in a mouse model of DM-2, and neurons from these animals were more vulnerable against hypoxia in vitro, compared to normal neurons (Navaratna et al., 2011).

2. Results

No animal died before the evaluation of volumes of infarcted lesions in the acute and chronic phase studies. During the operation, the physiological parameters of mice were stable and regulated within the normal range. There were no significant differences in body temperature, heart rate and mean arterial blood pressure between vehicle- and the three different AGL-treated groups during the operative period (Table 1). No significant differences were observed in body weight or blood glucose levels at the end of the treatment, with blood glucose levels of 170±22 mg/dl vs. 180±23 mg/dl in the vehicle- and AGL-treated groups respectively (p=0.234). Body weight was 23.5±1.1 g in the vehicle-treated vs.22.9±0.8 in the AGL-treated group (p=0.117).

2.1. The effect of prophylactic AGL treatment on infarcted lesion volumes and neurological deficits

On analysis of the volumes of infarcted lesions, a significant reduction was observed in Group III (medium dose), as compared to group I (vehicle) (Fig. 1A and B). There was no significant difference in the edema index between the groups (data not shown).

On assessment of neurological function in the acute phase (Fig. 1C), the SND score in group III was significantly smaller compared to group I (Mann-Whitney test), with no other differences.

In the chronic phase, the volume of infarcted lesion in group II (medium dose) was significantly smaller compared to those in group I (vehicle) (Fig. 2A and B). The SND score was significantly smaller in group II during the first two days, but with gradual improvements in both groups, the difference diminished.

2.2. Alterations in regional cerebral blood flow (rCBF) after the prophylactic treatment with AGL

There was no significant difference in the rCBF between the AGL (medium dose) and vehicle groups during ischemia (Fig. 3). After reperfusion, rCBF levels remained higher in the AGL group, achieving statistical significance during the later phase.
2.3. Determination of BDNF levels after the prophylactic AGL treatment

The BDNF levels in the whole forebrain were significantly elevated in the AGL group compared with the vehicle group (Fig. 4). In the forebrain, there were significant elevations in the cortex, and the thalamostriatum. The BDNF levels in the hippocampus did not achieve a significant difference.

2.4. The effect of post hoc AGL treatment on volumes of infarcted lesions and neurological deficits

On analysis of the volumes of infarcted lesions in the acute phase (Fig. 5A), the reduction in the AGL (medium dose)-treated group did not achieve a significant difference, as compared with vehicle alone (Fig. 5B). There was no significant difference in the edema index between the groups (data not shown). In the chronic phase, the volumes of infarcted lesions were not different between the groups (Fig. 5B). On assessment of neurological function, the SND score was not different between the groups, for seven days after ischemia (Fig. 5C).

3. Discussion

It was demonstrated that chronic, prophylactic treatment with AGL increased BDNF levels in the brain, and protected the brain against ischemic stroke. The pharmacokinetics and the efficacy profiles of AGL on glucose/insulin/glucagon levels in plasma after acute or chronic administration have been extensively studied in diabetic and normal animals (Moritoh et al., 2008; Lee et al., 2008), with a mean half-life of 3.6 h in normal rats, and 28 h in normal monkeys. After a single gavage (0.5 mg/kg) of AGL in normal rats, maximum inhibition (90%) of DPP-4 occurred at 30 min, which declined to 40% at 12 h, and disappeared within 24 h (Lee et al., 2008). We discontinued the treatment 24 h before the onset of ischemia to exclude, or at least minimize, any direct effects of AGL on cerebral ischemia.

It is well known that hyperglycemia is an exacerbating factor in ischemic stroke in patients with DM-2. However, normal blood glucose levels were not reduced by chronic, prophylactic treatment with AGL. AGL actually has only a minor effect on individuals with normal blood glucose levels. Administration of extremely high doses of AGL (100 mg/kg) showed no effect on fasting plasma glucose or insulin levels in normal mice (Lee et al., 2008), confirming that the effects of AGL on insulin secretion and insulin resistance are dependent in the presence of hyperglycemia.

Functional deterioration improved in both the chronic AGL- and vehicle-treated groups on entering the chronic phase, obliterating the initial difference between the groups. Because the rate of passage of biological time correlates inversely to [body weight]², as represented by longevity and heart/respiration rate (Calder, 1983), 15–30 min in mice is regarded as from 30 min to 1 h in rats (Yanamoto et al., 2004), and 3–6 h in humans (Yanamoto et al., 2012). Seven days after ischemia in mice can be translated into three months in humans, a sufficient time for a mild-to-moderate neurological deficit to improve. However, it is possible that the SND scoring system may not be sensitive enough to detect subtle differences in the chronic phase.

In the late reperfusion period, rCBF was higher in the AGL-treated group (Fig. 3). We speculate that the brain damage during ischemia was more severe in the vehicle group, which
brought on more severe cerebral edema during reperfusion, and reduced the rCBF, as demonstrated in our previous studies (Yamamoto et al., 2008, 2011). Although treatment of mice with AGL may upregulate endothelial nitric oxide synthase (eNOS) (Ban et al., 2008), rCBF was not increased during ischemia.

In DM-2 rats, treatment with Ex-4 (0.1, 1 or 5 μg/kg, via intraperitoneal injections, twice a day), before (for four weeks) and after (for two or four weeks) the induction of focal ischemia, reduced hyperglycemia and the volumes of infarcted lesions in a dose-dependent manner (Darsalia et al., 2012). In normal rats, prophylactic treatment with Ex-4 (0.5 μg/kg, via intraperitoneal injections, twice a day) for seven days reduced volumes of infarct lesion, the extent of neurological deficits, and also markers of oxidative stress (Briyal et al., 2012). Recently, intravenous injection of Ex-4 (0.5 or 2.5 mg/kg, immediately, or 1 h after the induction of reperfusion) reduced the volumes of infarcted lesions and

Fig. 1 – Comparisons of cerebral infarction and SND scores after ischemia in the acute phase. (A) Representative images of coronal sections (front views) of mouse brains 24 h after ischemia. (B) Quantification of volumes of infarcted lesions. (C) Comparisons of SND scores in vehicle- and AGL (low, medium, high dose)-treated groups.
the extent of functional deficits, without altering plasma insulin or glucose levels, in non-diabetic C57BL/6 mice (Teramoto et al., 2011).
The dose used was than was the case with effective prophylaxis against ischemia using Ex-4 (Darsalia et al., 2012; Briyal et al., 2012), with the same dose used for effective prophylaxis with AGL; (2) Ex-4 acts as a long-acting analog of GLP-1, while AGL increases intrinsic GLP-1; (3) Ex-4 was given intravenously, in contrast to the intragastric gavage used to administer AGL; (4) the intraluminal thread insertion (ITI) method (a 60-min focal ischemia) was used to assess volumes of infarcted lesions with Ex-4, but the three-vessel occlusion (3-VO) method (a 15-min focal ischemia limited in the cortex) was used with AGL. Considering the difference in biological time, 15-min delay (plus the delay for the transfer into the brain) after the onset in mice could be translated into more than 3-h delay in humans. Further investigations are needed, in which AGL is administered immediately, or within 15 min after the onset of ischemia.

Ischemia induces abnormal release, from 5- to 50-fold elevations, of glutamate and gamma-aminobutyric acid (GABA) in the brain (Matsumoto et al., 1996). The enhanced glutamate release has been reported to be neurotoxic (Mattson, 2008), and the enhanced GABA release to be neuroprotective (Pamenter et al., 2011; Zhou et al., 2008; Costa et al., 2004). As regards the mechanism by which BDNF protect the brain against cerebral ischemia, a chronic increase in BDNF levels increases the number of GABAergic synapses (Hong et al., 2008), and enhances the likelihood of GABA release (Baldelli et al., 2005). Therefore, a chronic increase in BDNF levels in the brain can act as a neuroprotectant by increasing GABA release during ischemia.

Regarding differential efficacy among the treated groups, a medium dose of AGL alone – a dose equivalent to the standard dose for treatment of human DM-2 – displayed an evident reduction in volumes of infarcted lesions. Administration of a DPP-4 inhibitor, sitagliptin, with an excessive dose (100 mg/kg/day, i.e. 50–100 times larger than the effective dose used for human DM-2) for 12 weeks, paradoxically increased tau phosphorylation in the hippocampus of DM-2 rats (Kim et al., 2012). It has also been shown that excessive BDNF levels impair learning and memory (Nakajo et al., 2008; Yanamoto et al., 2008). Although the mechanism is unknown, excessive doses may be ineffective or unsafe when DPP-4 inhibitors are used as neuroprotectants or a neurotrophins.
Although AGL treatment for three weeks did not induce significant weight loss in normal mice \( (p = 0.117) \), increased BDNF in the brain has the ability to normalize excessive appetite and obesity \( (Tsao et al., 2007; Nakagawa et al., 2003) \). Further investigations are needed to clarify whether AGL treatment may be a good choice for the risk reduction of brain injury due to an enhancer of BDNF production in the brain, aiming to halt appetite and obesity \( (Tsao et al., 2007; Nakagawa et al., 2003) \). In summary, AGL might be useful as a neuroprotectant, or an enhancer of BDNF production in the brain, aiming to halt minimization of brain injury due to first-ever or recurrent ischemic stroke.

4. Experimental procedures

This protocol of study was approved by the Animal Care and Use Committee of the NCVC. Every effort was made to minimize both the number of animals used and their suffering. In the assessment of infarcted lesions, BDNF levels in the brain or rCBF, sample sizes were calculated to detect a 30–35% alteration with 95% confidence considering the corresponding mean and the standard deviation (S.D.) in our previous studies \( (Yuan et al., 2009) \). We used computer-generated randomization schedules for the randomization of experimental animals. By using our three-vessel occlusion (3VO)-technique for the induction of temporary focal ischemia, there was no need to make selection criteria and exclude animals \( (Yamamoto et al., 2003) \).

The induction of ischemia and the assessment of volumes of infarcted lesions or neurological deficits were performed by a trained neurosurgeon who was blind to the treatment. The drug (or vehicle) administration and the monitoring of physiological parameters (body weight and blood glucose) during the treatment were performed by an independent investigator.

4.1. Analysis in the acute phase on the AGL treatment performed before ischemia

Male C57BL/6 J mice (8–11 weeks old, Japan CLEA, Japan) were randomly divided into the following four groups \( (n = 10/\text{group}) \) for the administration of AGL (purchased from Takeda Pharm. Co. Ltd., Japan) or vehicle. Doses were determined based on the human clinical dose \( (Scott, 2010) \): Group I, vehicle (saline); group II, low-dose AGL \( (7.5 \mu g/\text{day} = 0.25 \text{mg/kg/day}) \); group III, medium-dose AGL \( (15 \mu g/\text{day} = 0.5 \text{mg/kg/day}) \); and group IV, high-dose AGL \( (30 \mu g/\text{day} = 1.0 \text{mg/kg/day}) \). Saline, or AGL dissolved in 0.2 ml saline was administered once a day for three consecutive weeks via intragastric gavage. After treatment, mice were subjected to the brain surgery to induce temporary focal ischemia. Neurological deficits and the volumes of infarcted lesions were analyzed 24 h after ischemia.

4.2. Analysis in the chronic phase on the prophylactic AGL treatment

A second cohort of mice was randomly divided into the following two groups: Group I, vehicle (saline); group II, AGL \( (0.5 \text{mg/kg/day}) \) \( (n = 11/\text{group}) \), with a dose that was determined based on the results of the acute-phase analysis. The timing and nature of the surgery that was used to induce ischemia were exactly as above. Neurological deficits were assessed daily, and the volumes of infarcted lesions were analyzed seven days after ischemia.

4.3. Analysis on the AGL treatment post hoc

A third cohort of mice \( (n = 52) \) was randomly divided into the following two groups: Group I, vehicle (saline); group II, AGL \( (0.5 \text{mg/kg/day}) \). The administration of AGL or vehicle was performed immediately after the induction of reperfusion (after the insult of 15-min temporary focal ischemia as described below), once via intragastric gavage. Neurological deficits were assessed daily, and the volumes of infarcted lesions were analyzed 24 h or seven days \( (n = 13/\text{group}) \) after ischemia.

4.4. Induction of ischemia

Temporary, focal ischemia was produced in the left neocortex using the 3VO technique \( (Yamamoto et al., 2003, 2008, 2011; Nakajo et al., 2008) \). Briefly, the left middle cerebral artery (MCA) at the location distal to the lenticostriate arteries, the lateral edge of the olfactory tract, was cauterized. Bilateral common carotid arteries (CCAs) were simultaneously clip-occluded at the neck for 15 min, under surgical microscope with halothane-inhalation anesthesia and the monitoring of vital signs.

4.5. Physiologic parameters

During the anesthesia, rectal temperature was regulated within the physiological range, at \( 37 \pm 0.5 \text{°C} \), before, during, and after ischemia. Heart rate and mean blood pressure were monitored via the proximal tail artery. Blood glucose levels were analyzed at the same time during the day (from 11 to 12 A.M.).

4.6. Evaluation of neurological deficits

24 h (in the acute phase), or for 7 days (in the chronic phase), after the induction of ischemia, the functional consequences induced by ischemic stress and cerebral infarction were examined according to our original stroke-induced neurological deficit (SND) score \( (Yamamoto et al., 2001, 2011) \). Balance in the body trunk while being lifted by the tail was graded according to the following criteria: 0, no deficit (no twisting of the body); 1, mild deficit (asymmetric twisting tendency of the body); and 2, severe deficit (repeated asymmetric twisting of the body). Motor function of the extremities while being lifted by the tail was graded as follows: 0, no deficit (symmetrical movement of the forelimbs); 1, mild deficit (intermittent asymmetrical flexion of the forelimbs); and 2, severe deficit (continuous asymmetrical flexion of the forelimbs). The SND score (from 0 to 4) comprises the sum of the grades of the balance in body trunk and motor function of extremities.
4.7. **Measurement of cerebral edema and infarcted lesion volumes**

The volumes of infarcted lesions were analyzed at 24 h (in the acute phase), or seven days (in the chronic phase) after ischemia. Mice were perfused transcardially with heparinized PBS at 24 h or seven days after the induction of ischemia to washout any blood components from the brain tissue. The brain was removed and cut from the frontal tip into 1-mm thick coronal slices. Viable tissue was stained red with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Bederson et al., 1986), followed by fixation with 4% paraformaldehyde in PBS. The infarcted lesions and total hemispheric areas of each slice were measured by tracing the borders in a computer-assisted image-analysis system WinROOF (Mitani Co. Ltd.). In the acute phase alone, an edema index was calculated as the volume of the left hemisphere divided by the volume of the right hemisphere. The infarct index was calculated as the infarction volume divided by the edema index, which represents the actual infarcted lesion (dead tissue) volume, excluding any enlargement due to cerebral edema.

In the assessment of the chronic phase, the volume of infarcted lesion was calculated as the volume of the right (intact, residual) cortex minus the volume of the left (normal) cortex, which includes the volume of acute necrosis plus delayed cerebral atrophy (Yamamoto et al., 2011). We utilized TTC method that visualizes survived cells both in the acute and chronic phase for a chronological comparison, rather than utilizing the cresyl violet method that stains survived neurons. It was found that the brain tissue including degenerating and necrotic tissues shrank down to 66% of the original volume, in average, after the dehydration procedure needed in the cresyl violet method (Yamamoto et al., 1999). Proliferated reactive astrocytes (gliosis) in the border zone of focal ischemia, which is stained with glial fibrillary acidic protein (GFAP) or TCC, was negligible in the analysis of infarcted volumes in the cortex, because gliosis developed primarily in the corpus callosum, under the cortex (Yamamoto et al., 1999).

4.8. **Determination of regional cerebral blood flow**

A forth cohort of mice was randomly divided into the following two groups: treated with medium-dose AGL; or vehicle (N=11/group). The reduction and recovery levels of rCBF, before (control), during and after 3VO-ischemia were monitored using the laser-Doppler blood flowmetry meter TBF-LN1 (Unique Medical) (Yamamoto et al., 2011). The ROI was set in the MCA territory peripheral to the ischemic core; at 2 mm caudal and 1 mm dorsal to the occlusion point of the MCA. The values were expressed as percentages of the pre-ischemic baseline value in each animal.

4.9. **Quantitiation of BDNF level**

In the cohort of mice treated with medium-dose AGL (N=7), or vehicle (N=8), after trans-cardiac, pressure-regulated perfusion with PBS, cerebral neocortex, basal ganglia, and hippocampus were removed and kept frozen at –80°C till analysis. The brain tissue was homogenized in buffer, and the BDNF protein levels were determined with the two-site sandwich ELISA kit (Emax Immunoassay System, Promega, USA). BDNF levels were normalized by the amount of protein in each sample. The protein concentration was measured using a BCA Protein Assay kit (Thermo Scientific, USA). All assays were performed in triplicate.

4.10. **Statistical analysis**

All data are presented as the means±standard deviation (S. D.). One-way ANOVA with the post-hoc Holm-Sidak method was applied to compare the variance within the different parameters. The SND scores were examined by the non-parametric Mann–Whitney test at each time point. A p-value <0.05 was considered to be statistically significant.

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