The specific VPAC2 agonist Bay 55-9837 increases neuronal damage and hemorrhagic transformation after stroke in type 2 diabetic rats

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A B S T R A C T

VPAC2 receptor is a potential target for the treatment of type 2 diabetes and may also convey neuroprotective effects. The aim of this study was to determine the potential efficacy of the VPAC2 receptor agonist Bay 55-9837 against stroke in type-2 diabetic Goto-Kakizaki (GK) rats. GK rats were treated intravenously once daily for 7 days with 0.25 or 0.025 nmol/kg Bay 55-9837 or vehicle before inducing stroke by transient middle cerebral artery occlusion. Treatments were then continued for 7 further days. The glycemic effects of Bay 55-9837 were assessed by measuring fasting blood glucose and oral glucose tolerance. The severity of stroke was measured by assessing ischemic volume. The results show that Bay 55-9837 is not effective in lowering fasting glycemia and does not facilitate glucose disposal. The highest dose of Bay 55-9837 (0.25 nmol/kg) led to increased mortality and brain hemorrhage when compared to control. The lower dose of Bay 55-9837 (0.025 nmol/kg) did not increase mortality rate but caused a threelfold increase of the ischemic lesion size with signs of brain hemorrhages as compared to control.

In conclusion, Bay 55-9837 did not show antidihabetic or antistroke efficacy in the type 2 diabetic GK rat. Contrarily, Bay 55-9837 treatment led to increased mortality and worsening of the severity of stroke.

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1. Introduction

The incidence of stroke is elevated several-fold in diabetic patients, and is associated with a dramatically increased stroke severity and recurrence rate (Gaede et al., 2003; Haratz and Tanne, 2011; Kruyt et al., 2010; Zimmet et al., 2001). In fact, macroangiopathy (stroke and myocardial infarction) is the cause of death in 75–80% of type 2 diabetic patients (Sjoholm and Nystrom, 2006). Such increase in stroke incidence and severity in diabetes call for the development of therapies that manage diabetes and at the same time mitigate the risk or the consequences of stroke. Additionally, stroke patients with acutely deranged cerebral circulation are vulnerable to the profound metabolic stress evoked by hypoglycemia (Kruyt et al., 2010). Hence, hypoglycemia should be avoided in diabetic patients with stroke and insulin clearly is not the best drug in that respect. Thus, the diabetic population is in high need for novel drugs that exert robust anti-diabetic properties without hypoglycemic side effects, and that ideally also show neuroprotective effects perse.

The vasoactive intestinal peptide (VIP) receptors are interesting targets for accomplishing this dual role. VIP is a 28-amino acid neuromedin family (Dickson and Finlayson, 2009), which also includes pituitary adenylate cyclase-activating polypeptide (PACAP) (Arimura, 1998). VIP and PACAP share 67% amino acid sequence. The effects mediated by VIP and PACAP are pleiotropic and both peptides have been shown to regulate the physiology of the digestive tract, the cardiovascular system, the airways, the reproductive system, the immune system and the brain (Fahrenkrug, 2010; Makela et al., 2010; Moody et al., 2011; Takei et al., 2000; Vaudry et al., 2009). While both VIP and PACAP can bind to both VIP1 and VIP2 receptor subtypes [which were re-named VPAC-1 and VPAC-2 receptors, respectively, by the XVIII international union of pharmacology (Harmar et al., 1998)], PACAP also acts via a third receptor, PAC-1 (Dickson and Finlayson, 2009). PAC-1 receptors are particularly abundant in the brain, pituitary and adrenal gland, while VIP receptors are expressed mainly in lung, liver and testis (Vaudry et al., 2009) and less abundantly in the brain (Fahrenkrug, 2010; Mansouri et al., 2012; Mutt and Said, 1974; Shneider et al., 2010).

Neuroprotection through PAC-1 signaling is well documented (Brenneman, 2007; Ohtaki et al., 2006; Reglodi et al., 2000), and PAC-1 expression levels are increased in pathological states, such as ischemic brain injury (Shioda et al., 2006). Neuroprotective actions of VPAC-1 and VPAC-2 receptor agonist have also been reported in neurological disorders such as brain trauma, Parkinson’s and Alzheimer’s (Nishimoto et al., 2011; Rangon et al., 2005; Reglodi et al., 2011; White et al., 2010). In addition, a neuroprotective role...
mediated by VIP against stroke has been recently shown (Yang et al., 2011). With regard to mechanisms regulating VIP/PACAP receptor-mediated neuroprotection, PAC-1 has been shown to mediate direct neuronal surviving effects (Ohtaki et al., 2006; Reglodi et al., 2000).

In addition, anti-neuroinflammatory properties, indirectly leading to neuroprotection, have been associated with each of the three VIP/PACAP receptors (Delgado et al., 2008; Kim et al., 2000; Nishimoto et al., 2011; Tan and Waschek, 2011), where a potent microglia-deactivating action in the production of proinflammatory factors was reported (Ganea et al., 2003).

Bay 55–9837 is a potent VPAC2 receptor agonist that has been suggested to be useful in the treatment of type 2 diabetes (Tsutsumi et al., 2002). This compound stimulates glucose-dependent insulin secretion and synthesis in rat and human pancreatic islets and increases plasma insulin levels in non-diabetic rats (Tsutsumi et al., 2002). Furthermore, fasting hypoglycemia was not observed following Bay 55–9837 treatment in vivo (Tsutsumi et al., 2002). We hypothesized that VPAC2 activation by Bay 55–9837 has high potential for the treatment of stroke in diabetes. This hypothesis was based on the reported neuroprotective effects mediated by VPAC2 activation in combination with the Bay 55–9837-mediated anti-diabetic properties. Furthermore Bay 55–9837 is not prone to producing hypoglycemic effects that are deleterious for the brain after a stroke. Thus, we here have studied the potential antistroke efficacy of Bay 55–9837 in type 2 diabetic middle-aged Goto-Kakizaki (GK) rats (Ghanaat-Pour and Sjoholm, 2009; Suzuki et al., 1990).

2. Methods

2.1. Drug preparation

Bay 55–9837 was obtained from Tocris Bioscience, Bristol, U.K. The drug was dissolved in PBS containing 0.1% BSA, frozen in aliquots, kept at −20 °C and thawed immediately before use.

2.2. Animals and experimental groups

Thirty-seven 7–9 month-old male diabetic GK rats were used. The GK rat is a non-obese Wistar sub-strain, which spontaneously develops insulin resistance and type 2 diabetes in early life (Ghanaat-Pour and Sjoholm, 2009). 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. Before the start of the Bay 55–9837/vehicle treatment, baseline fasting blood glucose concentrations were measured and the animals were assigned to the different treatment groups so that mean blood glucose values were equalized. Treatment groups thus created were tested for normality using the D’Agostino & Pearson omnibus normality test. All rats received intravenous (via the tail vein) injections of 0.25 (n = 6) or 0.025 (n = 16) nmol/kg bw Bay 55–9837 or vehicle (n = 19) once daily for 7 days before being subjected to stroke. Bay 55–9837 and PBS injections were continued for another 7 days following the stroke until the animals were sacrificed.

2.3. Glycemia and oral glucose tolerance test

The levels of fasting blood glucose were measured in diabetic GK rats after 1 week of daily i.v. injections of 0.25 nmol/kg bw Bay 55–9837 or vehicle. After initial blood glucose measurements, the rats were given 1.5 g/kg bw glucose orally and blood samples were taken after 5, 15, 30, 60, 90 and 120 min. Oral glucose tolerance test was performed before stroke and blood glucose levels were measured both before and during stroke.

2.4. Middle cerebral artery occlusion (MCAO)

Rats were anesthetized by spontaneous inhalation of 1.5% isoflurane through a snout-mask. Body temperature was maintained at 37–38 °C using a heating pad. Stroke was induced by the intraluminal filament technique (Koizumi et al., 1986). Briefly, common and external carotid arteries were ligated, and the internal carotid artery was temporarily closed. A 0.22 mm monofilament was advanced through the internal carotid artery to the origin of the middle cerebral artery (MCA), the wound was closed, and the animal was allowed to wake up and was placed in its home cage. After 90 min of occlusion, the animal was anesthetized again and the filament was withdrawn. The surgeon performing the operation was blinded to the treatment groups.

2.5. Immunocytochemistry

Animals were deeply anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer. The brains were post-fixed in 4% paraformaldehyde overnight and submersed in 20% sucrose in phosphate buffer until they sunk. Forty μm-thick coronal sections were cut using a sliding microtome and stained as free-floating sections. Mouse anti-NeuN, a specific neuronal marker (1:300; Millipore, Billerica, MA) was used to stain the sections. Sections were incubated with primary antibody overnight at 4 °C in phosphate buffer containing 3% normal horse serum and 0.25% Triton-X, followed by biotinylated horse anti-mouse secondary antibody for 2 h at room temperature in phosphate buffer containing 3% normal horse serum and 0.25% Triton-X. For chromogenic visualization, avidin–biotin complex (ABC kit, Vector, CA) and diaminobenzidine were used.

2.6. Tissue damage evaluation and cell quantification

Neuronal damage was assessed by an investigator blinded to the experimental groups. 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 to stroke tissue were measured in every section containing stroke damage using Newcast software (Visiopharm, Hoersholm, Denmark). To compensate for the stroke-induced morphological tissue changes, the infarct volume was calculated by subtracting the volume of remaining tissue in the ipsilateral hemisphere from the volume of the contralateral hemisphere.

2.7. Statistics

Statistical analyses were performed using unpaired t-test or repeated measures two-way analysis of variance (ANOVA), followed by Bonferroni’s post hoc test. Differences between groups were considered statistically significant when P < 0.05. Data are presented as means ± SEM.

3. Results

3.1. Intravenous administration of Bay 55–9837 has no effect on fasting glycemia or glucose tolerance in GK rats

No difference was observed between the groups in fasting blood glucose levels after 1 week of Bay 55–9837 or vehicle treatment (Fig. 1A) or glucose disposal after OGTT (Fig. 1B). Non-fasting glucose levels were also measured during MCAO surgery with no
significant difference between the treatment groups (Fig. 1C). No statistically significant change in blood glucose levels between fasting and non-fasting states were observed within or between the experimental groups.

3.2. Bay 55-9837 administration increases mortality, intracranial hemorrhages and worsens the stroke outcome

Five out of six GK rats treated with 0.25 nmol/kg Bay 55-9837 died prematurely within 24 h after MCAO. One animal survived until the end of the experiment. As post mortem examination revealed that all 6 animals had visible intracranial hemorrhages. For the next series, the dose of Bay 55-9837 was decreased 10 times, and twelve GK rats thus received 0.025 nmol/kg Bay 55-9837. As a result, the mortality rate decreased to 33% (4 out of 12), but the incidence of intracranial hemorrhages remained high (83%, 10 out of 12). In PBS-treated GK rats we observed 26% mortality (all due to intracranial hemorrhages), and no hemorrhages were detected in animals that survived until the end of the experiments. The rate of mortality and intracranial hemorrhages are summarized in Table 1.

The infarct volume was measured in stroke-subjected GK rats after the treatment with Bay 55-9837 or vehicle. The infarct volume was significantly greater in Bay 55-9837-treated GK rats than in vehicle-treated animals (Fig. 2).

4. Discussion

VIP and PACAP are pleiotropic factors which have been shown to exert, among others, neuroprotective, anti-inflammatory and anti-diabetic effects (see Introduction). However, the therapeutic potential of these neuropeptides has been questioned due to their cardiovascular side effects, example, vasodilatation and hypotension (Grant et al., 2006; Ishizuka et al., 1992; Nandha et al., 1991) as well as to their involvement in the pathophysiology of migraine (Witte et al., 2002) and diarrhea (Modlin et al., 1978). Thus, to develop VIP/PACAP agonists into therapeutics, it becomes important to identify specific ligands for these receptors that bear the therapeutic properties without showing major side effects.

Bay 55-9837 is a specific VPAC-2 receptor agonist that has been proposed for the treatment of type 2 diabetes (Tsutsumi et al., 2002). Interestingly, Bay 55-9837 does not influence intestinal water retention and arterial blood pressure except at very high doses (Tsutsumi et al., 2002). Furthermore, while PACAP and its PAC-1 receptor-selective agonist maxadilan have been shown to increase heart rate (Hoover et al., 2009), Bay 55-9837 does not (Tsutsumi et al., 2002). In view of the growing need for therapeutics against stroke, we considered Bay 55-9837 as a drug with therapeutic potential. This hypothesis was supported by the reported lack of hypoglycemia following Bay 55-9837 administration in normal Wistar rats (Tsutsumi et al., 2002). A further impetus was our findings of high levels of VPAC-2 receptor expression in striatum (Mansouri et al., 2012), a brain area that is primarily damaged in our model of stroke and frequently also in humans suffering a stroke.

In this study, we started with 0.25 nmol/kg Bay 55-9837, since this dose was reported to be highly efficacious in stimulating insulin secretion in vivo (Tsutsumi et al., 2002). Furthermore, this dose of Bay 55-9837 did not, or only minimally, affects intestinal water retention and mean arterial pressure (Tsutsumi et al., 2002). However, our results with 0.25 nmol/kg Bay 55-9837 did not show improved fasting glycemia or glucose tolerance in type 2 diabetic GK rats. A possible explanation for this discrepancy is that we employed a type 2 diabetic animal model, while the work of Tsutsumi et al. was performed in non-diabetic Wistar rats (Tsutsumi et al., 2002). The discrepancy between the latter results and our findings may be due to the impairment of beta-cell insulin secretion that characterizes the GK model (Ghanaat-Pour and Sjoholm, 2009). In short, our results do not support VPAC-2 receptor activation by Bay 55-9837 as a potential treatment of type 2 diabetes.

A very high mortality due to severe brain hemorrhages was observed following stroke combined with 0.25 nmol/kg of Bay 55-9837, as compared to controls. We therefore reduced the Bay 55-9837 dose 10 times and repeated the stroke experiments. The main result was a 2–3 times larger ischemic lesion, visualized by NeuN staining, in the Bay 55-9837-treated group as compared to controls. However, there was a significant reduction of mortality rate in animals treated with low vs. high doses of Bay 55-9837. Interestingly, the low dose of Bay 55-9837 employed in our study was five times lower than the lowest dose of Bay 55-9837 reported to minimally impact the mean arterial pressure in Wistar rats (Tsutsumi et al., 2002).

Table 1

<table>
<thead>
<tr>
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<th>N</th>
<th>Hemorrhagic transformation</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bay 55-9837 (0.25 nmol/kg)</td>
<td>6</td>
<td>6 (100%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>Bay 55-9837 (0.025 nmol/kg)</td>
<td>12</td>
<td>10 (83%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>PBS</td>
<td>19</td>
<td>5 (26%)</td>
<td>5 (26%)</td>
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Recent results by Yang et al. showed efficacy of VIP against stroke in a non-diabetic rat model subjected to stroke in a similar manner as in our study (Yang et al., 2008). Although we cannot rule out the possibility that the efficacy shown by Yang et al. was mediated by VPAC1 activation, VIP was delivered intracerebroventricularly in this study. Thus, it is possible that the reported efficacy was still mediated by direct VPAC2-mediated neuroprotection, although “by passing” those systemic side effects that led to brain hemorrhage in our study.

As with several side effects of Bay 55–9837, except at very high doses, were reported by Tsutsumi et al. (Tsutsumi et al., 2002), the high rate of mortality and hemorrhages in our study could possibly be attributed to the combination of the following factors: the diabetic GK rats develop severe endothelial dysfunction over time, which leads to decreased arterial relaxation (Kazuyama et al., 2009) and increased blood pressure (Witte et al., 2002). GK rats have been reported to exhibit significant alterations in cerebral vasculature (Beauquis et al., 2010), which-together with the above hypertensive–may underlie their increased propensity for hemorrhagic transformations after stroke (Ergul et al., 2007). Thus, imposing vasodilation and hypotension by VPAC-2 receptor activation (Grant et al., 2006; Ishizuka et al., 1992; Nandha et al., 1991) to the brittle state of GK rat vasculature could have contributed to the negative effect on survival and stroke outcome in our study. Finally, since VIP has been previously reported to potently inhibit platelet activation (Grant et al., 2006; Tsutsumi et al., 2002), although we cannot rule out the possibility that the efficacy shown by Yang et al. was mediated by VPAC1 activation, VIP was delivered intracerebroventricularly in this study. Thus, it is possible that the reported efficacy was still mediated by direct VPAC2-mediated neuroprotection, although “by passing” those systemic side effects that led to brain hemorrhage in our study.

In conclusion, our results show that daily systemic administration of the VPAC-2 receptor agonist Bay 55–9837 in type 2 diabetic GK rats, at a dose previously shown to induce insulin secretion, does not produce anti-diabetic efficacy. Furthermore, Bay 55–9837 administration did not confer neuroprotection after stroke but rather worsened the insult. Considering the high rate of stroke risk and the severity of stroke damage in type 2 diabetes, our results clearly do not encourage the systemic use of VPAC-2 receptor agonists for treatment of stroke in the diabetic population.

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**References**


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