The 11-amino acid peptide urotensin II (UII) was first recognised to be an important hormone in teleost fish in the early 1970s. In mammals, UII has been identified as a vasoactive peptide that binds to the orphan G-protein-coupled receptor 14 (GPR14). Several recent reports have revealed the powerful vasoconstrictive effect of UII, which is 8–110-fold that seen with endothelin-1. 

Experimental and clinical studies have revealed the increased expression of UII and GPR14 in animals with experimentally induced myocardial infarction and heart failure, as well as in patients with hypertension, atherosclerosis, and diabetes mellitus (DM). These findings suggest a potential role for UII in cardiovascular and renal diseases. Using a UII-specific receptor antagonist, UII was found likely to become a new target for the prevention and treatment of the diseases mentioned above. Furthermore, recent studies have demonstrated that UII has multiple roles in cardiovascular diseases, some of which are independent of blood pressure (e.g., trophic and mitogenic actions).

Diabetes mellitus is a risk factor for cerebral ischaemia. The relative risk of cerebral ischaemia in diabetic patients has been shown to be approximately twice as high as in patients without DM. In addition, DM is strongly associated with early brain injury and with poor outcome after cerebral ischaemia.

Therefore, in the present study, expression of UII and its receptor in the diabetic ischaemic brain and its association with functional and pathological changes were investigated using a streptozotocin (STZ)-induced model of DM and the middle cerebral artery occlusion (MCAO) model. We wished to ascertain if UII and GPR14 had important roles in DM combined cerebral ischaemia.
**MATERIALS AND METHODS**

**Ethical approval of the study protocol**

The Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (Harbin, China) approved the study design. The University Animal Care and Use Committee approved all these procedures.

**Animal treatment**

Eighty male Sprague–Dawley (SD) rats (180–220 g) were purchased from the Laboratory Animal Center of the Second Affiliated Hospital of Harbin Medical University. The SD rats were divided into four groups: normal control, DM, MCAO and DM/MCAO. Rats were fasted overnight before the induction of DM but had free access to drinking water. DM was induced by a single intraperitoneal injection of 55 mg/kg STZ (Sigma–Aldrich, St. Louis, MO, USA) which was dissolved fresh in 0.1 mol/L citrate buffer at pH 4.5. The plasma level of glucose in all rats diagnosed as being diabetic was examined 72 hours (h) after STZ injection and was >16.7 mmol/L. Control rats were given an equal volume of 0.1 mol/L citrate buffer.

**MCAO model**

The MCAO model of DM was created as described previously. DM/MCAO was induced in the rats diagnosed as diabetic and age-matched control rats. The DM/MCAO model was implemented 30 days after STZ injection. All rats were killed six hours after cerebral ischaemia.

**Neurological evaluation**

Before killing, each rat was graded according to a six-point neurological scoring system for focal deficits, as described previously. That is: grade 0 denoted no apparent deficits; grade 1, flexion of the contralateral forelimb; grade 2, decreased grip of the contralateral forelimb while pulling of the tail; grade 3, spontaneous movement in all directions with contralateral circling only if the tail is pulled; grade 4, spontaneous contralateral circling; and grade 5, death. We selected grades 1–3 as the test measurements for these animals.

**Chinese ink perfusion model**

Before killing, rats were anaesthetised with an intraperitoneal injection of chloral hydrate (10%). The chest was opened and a cannula inserted in the aorta through the left ventricle. The distal aorta was clamped, the right auricle opened, and intravascular blood washed out with normal (0.9%) saline until clear fluid emerged from the right auricle. Then, 100–120 ml of Chinese ink was injected into the ascending aorta.

**Measurement of infarct size**

Rats were anaesthetised as described above and decapitated 6 h after MCAO. Brains were removed immediately and placed in ice-cold phosphate-buffered saline (PBS) for 15 minutes (min). Coronal sections of the brain were cut into 2-mm slices. Brain slices were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride monohydrate (in PBS, pH 7.4) at 37°C for 15 min, followed by 10% formaldehyde solution. The infarct area of each section was traced and quantified by an Metamorph image analysis system.

**Immunohistochemical (IHC) staining**

Brain-tissue sections of thickness 4 μm were used to conduct IHC staining for UII, GPR14 and vascular endothelial growth factor (VEGF) with the following specific antibodies: polyclonal goat anti-rat UII and anti-rat GPR14 antibodies, and monoclonal mouse anti-rat VEGF antibody (Sigma–Aldrich). Colour was developed by incubation with diaminobenzidine and counterstaining with hematoxylin. Controls were obtained by replacing the primary antibody with PBS. For semi-quantitative analyses, ten high-power microscope fields were selected randomly. The pathological image analysis system was used to calculate the percentage of positive staining.

**Reverse-transcription- polymerase chain reaction (RT-PCR)**

Total RNA was extracted from ischaemic brain tissues using TRIzol reagent (Gibco, Carlsbad, CA, USA). Primers for UII, GPR14, VEGF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and synthesised by Shanghai

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**Table 1: Upstream and downstream primers for UII, GPR14, VEGF and GAPDH**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence length</th>
<th>Length, bp</th>
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<tbody>
<tr>
<td>UII sense</td>
<td>5'-TGCCGTGCTCTTCGTAGGACT-3'</td>
<td>242</td>
</tr>
<tr>
<td>UII antisense</td>
<td>5'-AGAGCTTCTCCCAAGCTT-3'</td>
<td></td>
</tr>
<tr>
<td>GPR14 sense</td>
<td>5'-TCTGAGCTGGAGTCTACAACAAGCT-3'</td>
<td>351</td>
</tr>
<tr>
<td>GPR14 antisense</td>
<td>5'-CCAAAGTGCCAGTCCCTTAGTGACGT-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH sense</td>
<td>5'-ACCACAGTCCATGCCATCAC-3'</td>
<td>450</td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>5'-TCCACCAACCTGTTGCAGTG-3'</td>
<td></td>
</tr>
<tr>
<td>VEGF sense</td>
<td>5'-CTGCTCTCTCGGGTGACT-3'</td>
<td>200</td>
</tr>
<tr>
<td>VEGF antisense</td>
<td>5'-ATACACTATCTCATCGGGGTACT-3'</td>
<td></td>
</tr>
</tbody>
</table>

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Biological Engineering (Shanghai, China). The sequences for these primers are presented in Table 1. Total RNA (0.5 μg) was amplified using the Titan™ One Tube RT-PCR kit (Boehringer-Mannheim, Shanghai, China). Twenty-five cycles of replication were used. Bands were digitized using a Tanon-1000 Gel Image System (Shanghai). The ratios of UII, GPR14, or VEGF band density to GAPDH band density in various groups are presented.

**Statistical analyses**

Data are expressed as the mean ± SD. One-way analysis of variance (ANOVA) and Student’s t-test were used for statistical analyses. P<0.05 was considered significant.

**RESULTS**

**Neurological evaluation**

Neurological damage was scored 6 h after MCAO. Neurological dysfunction in the DM/MCAO group (2.83 ± 0.41) was increased significantly compared with that seen in the MCAO group (2.33 ± 0.51).

**Brain imaging after MCAO**

Compared with the brains of MCAO rats (Figure 1A), the brains of rats in the DM/MCAO group showed spontaneous cerebral haemorrhage (Figure 1B). Perfusion of Chinese ink revealed that vascular proliferation and branch thickening were more clearly observed in the DM/MCAO group (Figure 1D) compared with the brains of MCAO rats (Figure 1C).

**Infarct volume**

Representative samples of triphenyl tetrazolium chloride (TTC)-stained brain sections are shown in Figure 2. They demonstrated that, compared with rats in the MCAO group (Figure 2A), the infarction size (white-coloured areas) in the brains of DM/MCAO rats (Figure 2B) was smaller.

**Expression of UII and GPR14**

Examination of the proteins of UII and GPR14 by IHC staining suggested that expression of UII and GPR14 proteins were predominant in the cytoplasm of neurons and vascular endothelial cells (representative images of each group are presented in Figure 3). Semi-quantitative analyses (Figure 4) of the intensity and area of staining of UII, GPR14 and VEGF showed expression of UII and GPR14 was increased in the DM and MCAO group compared with controls. In the DM/MCAO group, expression of UII and GPR14 was increased significantly in the ischaemic brain compared with MCAO group, and was accompanied by a significantly increased VEGF expression. Furthermore, the increase in the expression of UII, GPR14 and VEGF proteins in brain was confirmed at the mRNA level by the RT-PCR assay, and the upregulated expression of UII in diabetic and ischaemic brain was accompanied by a significant increase in VEGF expressions (representative images of each group and semi-quantitative analyses of the intensity are shown in Figure 5).

**DISCUSSION**

Studies on cerebral ischaemia–reperfusion injury have shown that elevated levels of glucose result in injury to the ischaemic brain by provoking anaerobic metabolism, lactic acidosis; also, free-radical production and hyperglycemia may result in direct lipid peroxidation in the cell membrane. Levels of UII and GPR14 have been found to be higher in the blood and urine of DM patients. These findings suggest that DM is a reason for the
The effect of UII on ischaemia–reperfusion injury is controversial. One study focusing on the relationship between UII and ischaemia–reperfusion injury in the heart found that increased expression of UII and GPR14 can aggravate myocardial damage. Nevertheless, other results suggested that UII protects against ischaemia–reperfusion injury in hearts. The present study demonstrates that diabetes and focal cerebral ischaemia increased the expression of UII and GPR14. In the DM/MCAO group, expression of UII and GPR14 was increased significantly in the ischaemic brain compared with MCAO group, and was accompanied by a significantly increased VEGF expression. Semi-quantitative data for these proteins are summarized in Figure 4.

Vascular endothelial growth factor is an angiogenesis and vascular permeability factor that undergoes transcriptional and post-transcriptional induction by hypoxia; VEGF couples hypoxia to angiogenesis in diverse tissues (including the brain). Vascular endothelial growth factor may have an important role in the vascular response to cerebral ischaemia because ischaemia (i) stimulates VEGF expression in the brain and (ii) promotes the formation of new cerebral blood vessels. Vascular endothelial growth factor is one of the most important angiogenic cytokines involved in vascular remodelling, which can induce intimal hyperplasia in rabbit carotid arteries, and induce the proliferation and migration of adventitial fibroblasts.

The most important finding of the present study was that UII, GPR14 and VEGF were upregulated simultaneously in the brains of DM, MCAO and DM/MCAO rats. Urotensin II and angiotensin II (Ang II) are the two most important vascular peptides involved in vascular remodeling in the adventitia. UII is a weak nitric oxide (NO)-dependent vasodilator in the vasculature, is involved in the proliferation of vascular smooth muscle cells, and can induce vascular remodelling in the adventitia by inducing the differentiation, migration, proliferation of adventitial fibroblasts as well as collagen synthesis within them.

Recent studies have shown that UII can induce VEGF expression in adventitial fibroblasts and that VEGF is involved in UII- and Ang II-induced cell proliferation and collagen synthesis. It has also been demonstrated that VEGF-neutralizing anti-bodies can significantly inhibit Ang II- and UII-induced proliferation and collagen synthesis in adventitial fibroblasts, which suggests that VEGF may be a down-stream angiogenic mediator of Ang II and UII.

In this study, increased expression of UII and VEGF in the brain was mainly observed in the neurons and vascular endothelial cells simultaneously. We also found that the infarct volume in diabetic rats was significantly smaller in the DM/MCAO group than in the MCAO group, but exacerbates brain damage in the DM/MCAO group. We suggest that high levels of glucose promotes UII and induces the upregulation of VEGF expression that is conducive to preventing infarction and reducing the infarct volume. Vascular endothelial growth factor can induce the proliferation of vascular endothelial cells and smooth muscle cells and collagen synthesis in adventitial fibroblasts, thus inducing vascular remodelling, which increased cerebral blood flow and reduced the infarct volume. However, the cerebral vessels formed in response to VEGF are of abnormally poor stability and organisational structure; they
are often twisted, irregular and leaky. These features could exacerbate cerebral edema and haemorrhage, and worsen the outcome from ischaemia. Therefore, U-II may be an important factor in injury due to cerebral ischaemia under diabetic conditions. Our results are similar to what has been observed in anesthetized rats, Chuquet et al provide the first evidence that U-II increases cerebral blood flow and exacerbates brain damage following an ischemic insult. 

Increased expression of U-II induced by high levels of glucose promotes VEGF generation results in vascular remodelling, neovascularization and aggravated brain lesions, but its specific mechanism of action is incompletely understood. Therefore, further investigation is necessary to clarify the exact role of U-II in DM-aggravated brain lesions after ischaemia.

REFERENCES


