ORIGINAL ARTICLE

Expression of Urotensin II During Focal Cerebral Ischemic in Diabetic Rats

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ABSTRACT: ***Background:*** The objective of this study was to explore the expression of urotensin II (UII), its receptor (GPR14), and vascular endothelial growth factor (VEGF), as well as their associations in the ischaemic brains of rats with focal cerebral ischaemia, under normal and diabetic conditions. ***Methods:*** Diabetes mellitus (DM) was induced by injection of streptozotocin (STZ) into Sprague–Dawley rats. Focal cerebral ischaemia was induced by middle cerebral artery occlusion (MCAO) four weeks after DM onset by STZ. Rats (n=80) were divided into four groups: normal control, DM, MCAO, and DM/MCAO. Immunohistochemistry and reverse-transcriptase-polymerase chain reaction (RT-PCR) were used to detect the expression of UII, GPR14 and VEGF in the diabetic and ischaemic brain. ***Results:*** Expression of UII and GPR14 was increased at mRNA and protein levels 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, and was accompanied by a significantly increased VEGF expression. ***Conclusion:*** Diabetes mellitus was seen to aggravate brain lesions after ischaemia, and UII may have an important role.

RÉSUMÉ: Expression de l'urotensine II pendant une ischémie cérébrale focale chez des rats diabétiques. ***Contexte :*** Le but de cette étude était d'examiner l'expression de l'urotensine II (UII), de son récepteur (GPR14) et du facteur de croissance endothélial vasculaire (VEGF) ainsi que leur association dans l'ischémie cérébrale focale chez des rats normaux et des rats diabétiques. ***Méthode :*** Un diabète sucré a été induit par injection de streptozotocine (STZ) chez des rats Sprague-Dawley. Une ischémie cérébrale focale a été induite par occlusion de l'artère cérébrale moyenne (OACM) quatre semaines après le début du diabète induit par la STZ. Les rats (n = 80) ont été répartis en quatre groupes : témoins normaux, diabétiques, OACM et diabétiques/OACM. Nous avons utilisé l'immunohistochimie et l'amplification en chaîne par polymérase (RT-PCR) pour détecter l'expression d'UII, de GPR14 et de VEGF dans le cerveau des rats diabétiques et des rats ischémiques. ***Résultats :*** Les niveaux d'ARNm et de protéine d'UII et de GPR14 étaient augmentés dans le groupe diabétique et le groupe OACM par rapport au groupe témoin. Chez le groupe diabète/OACM, l'expression d'UII et de GPR14 était augmentée significativement dans le cerveau ischémique et ceci était accompagné d'une augmentation significative de l'expression de VEGF. ***Conclusion :*** Nous avons constaté que la présence de diabète aggrave les lésions après une ischémie et qu'UII pourrait y jouer un rôle important.

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The 11-amino acid peptide urotensin II (UII) was first recognised to be an important hormone in teleost fish in the early 1970s[1]. 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 vasocontractile effect of UII, which is 8–110-fold that seen with endothelin-1[2,3].

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)[4-7]. 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[6,8]. Furthermore, recent studies have demonstrated that UII has multiple roles in cardiovascular diseases[9-13], 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[14-16]. In addition, DM is strongly associated with early brain injury and with poor outcome after cerebral ischaemia[17,18].

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.

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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[19,20]. 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[19-22]. 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

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

Primer	Sequence length	Length, bp
UII sense	5'-TGCCTGCTCTTCGTAGGACT-3'	242
UII antisense	5'-AGAGCCTTCCTCAAGCTT-3'	
GPR14 sense	5'-TCTGAGCCTGGAGTCTACAACAAGCT-3'	351
GPR14 antisense	5'-CCAAAGTGCCAGTCCTTAGTGACGT-3'	
GAPDH sense	5'-ACCACAGTCCATGCCATCAC-3'	450
GAPDH antisense	5'-TCCACCACCCTGTTGC TGTA-3'	
VEGF sense	5'-CTGCTCTCTTGGGTGCACT-3'	200
VEGF antisense	5'-ATACACTATCTCATCGGGGTACT-3'	

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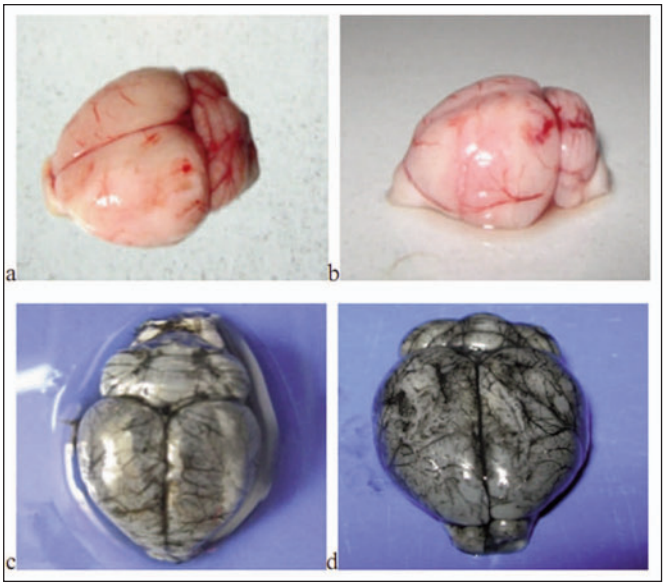

Figure 1: *Cerebral hemorrhage, vascular damage and its proliferation. The brain was examined for hemorrhage in MCAO (a) and DM/MCAO groups (b). Vascular damage in the brain was indicated by Chinese ink perfusion which showed that, compared with the brain of MCAO rats (c), there was evidence of vascular proliferation and branch thickening in the brains of rats in the DM/MCAO group (d).*

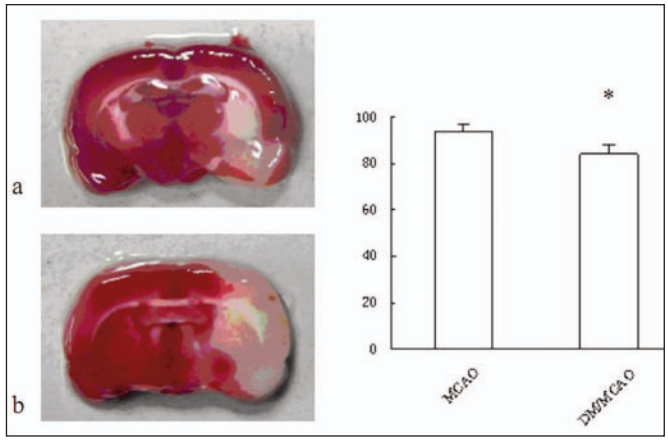

Figure 2: *Cerebral infarction. Upon examination 6 h after ischemia, DM/MCAO induced a significant infarction (a) that was relatively small compared with the infarction size in the MCAO group (b). Infarction was analysed semi-quantitatively as described in the Materials and Methods section. *P <0.05, versus the MCAO group (n=8).*

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

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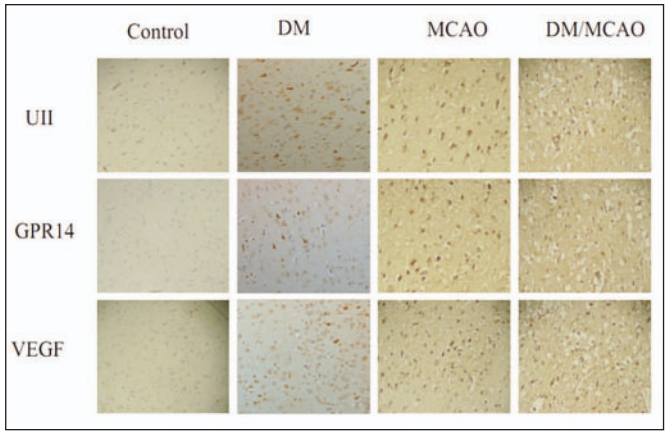

Figure 3: *Immunohistochemical staining. Animals were randomly divided into the following four groups: control, DM, MCAO, and DM/MCAO. Brain tissues were stained by immunohistochemical means for urotensin II (UII), G protein-coupled receptor 14 (GPR14), and vascular endothelial growth factor (VEGF). 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. Semi-quantitative data for these proteins are summarized in Figure 4.*

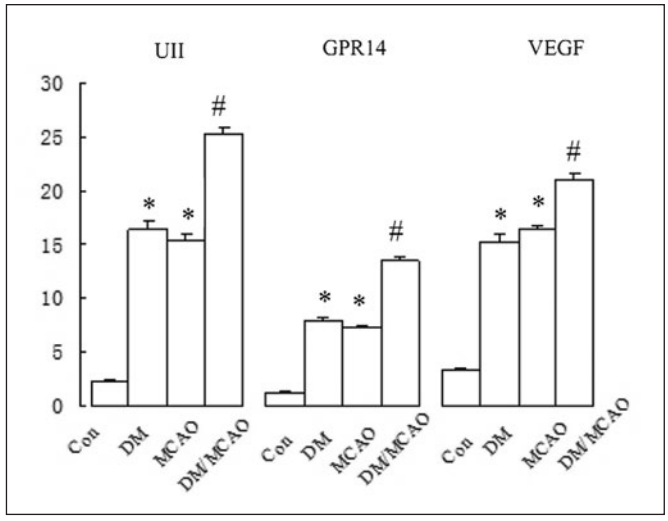

Figure 4: *Expression of UII, GPR14 and VEGF proteins in rat brains. Semi-quantitative data for these proteins. *P <0.05, versus control group; #P <0.05, versus MCAO group (n=8).*

elevation of UII levels in plasma, and it may be an important factor in cardiovascular problems in DM patients[7].

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[23]. Nevertheless, other results suggested that UII protects against ischaemia–reperfusion injury in hearts[24]. The present study demonstrats 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. We suggest that ischaemia was one reason for the increase in the expression of UII and GPR14, and that DM was another. In animal models of cerebral ischaemia, hyperglycemic animals have been shown to suffer greater neurological deficit with extensive brain damage and widespread necrosis than non-hyperglycemic animals[22,25,26].

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)[27]. 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[28] and (ii) promotes the formation of new cerebral blood vessels[29]. 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[30,31].

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[32]. UII is a weak nitric oxide (NO)-dependent vasodilator in the vasculature, is involved in the proliferation of vascular smooth muscle cells[33,34], and can induce vascular remodelling in the adventitia by inducing the differentiation, migration, proliferation of adventitial fibroblasts as well as collagen synthesis within them[35].

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[36]. 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[37], 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

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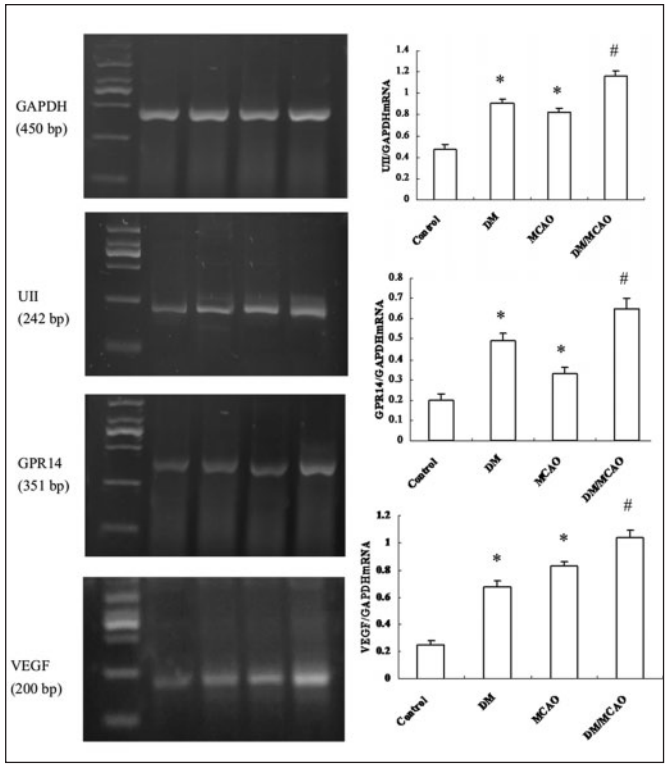

Figure 5: *Diabetes-induced upregulation of UII, GPR14 and VEGF mRNA expressions in rat brains. Brains of rats of different groups were collected at the times indicated to analyse UII, GPR14 and VEGF mRNA expressions by RT-PCR. Expression of UII, GPR14 and VEGF were increased in the DM and MCAO group compared with controls. In the DM/MCAO group, expression of UII, GPR14 and VEGF were increased significantly in the ischaemic brain compared with MCAO group.*P <0.05, versus control group; #P <0.05, versus MCAO group (n=8).*

are often twisted, irregular and leaky. These features could exacerbate cerebral edema and haemorrhage, and worsen the outcome from ischaemia. Therefore, UII 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[38].

Increased expression of UII 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 UII in DM-aggravated brain lesions after ischaemia.

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