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RESEARCH PAPER

Urocortin ameliorates diabetic nephropathy in obese *db/db* mice

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Background and purpose: Hyperglycaemia induces overproduction of mitochondrial reactive oxygen species (ROS) in endothelial cells, which is believed to be a major molecular mechanism underlying complications of diabetes, including diabetic nephropathy. Impairment of endothelium-dependent vasodilatation is found in type 2 diabetes. Urocortin is a 40 amino-acid peptide related to the corticotrophin-releasing factor (CRF) family, which suppresses production of ROS in endothelial cells and sustains endothelium-dependent relaxations of rat coronary artery. However, it is not clear if urocortin has any effect on diabetic nephropathy.
Experimental approach: Possible mechanisms underlying the effects of urocortin on diabetic nephropathy were investigated in *db/db* mice and cultured rat mesangial cells.
Key results: Urocortin decreased body weight, plasma levels of advanced glycation end-products, blood urea nitrogen and creatinine levels. However, food intake, plasma insulin and glucose levels remained unaffected. Superoxide dismutase activity was increased markedly, whereas malonaldehyde levels in kidney homogenate and sorbitol concentrations in red blood cells were decreased significantly in urocortin-treated mice. Urocortin significantly decreased glomerular extracellular matrix expansion and accumulation in kidney. Moreover, urocortin inhibited the overexpression of transforming growth factor-beta 1 and connective tissue growth factor in rat mesangial cells induced by 25 mM glucose. All the effects of urocortin, except sorbitol accumulation, were abolished by the non-selective CRF receptor blocker, astressin.
Conclusion and implications: Urocortin could significantly ameliorate diabetic nephropathy and this effect was mediated via the CRF receptor.
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Keywords: urocortin; diabetic nephropathy; extra cellular matrix

Abbreviations: AGE, advanced glycation end products; CRF, corticotrophin-releasing factor; CTGF, connective tissue growth factor; ECM, extracellular matrix; MC, mesangial cells; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β 1

Introduction

Diabetes mellitus is recognized as one of the leading causes of morbidity and mortality in the world. Diabetic nephropathy is one of the most severe diabetic microangiopathies and has become a worldwide epidemic, accounting for approximately one-third of all cases of end-stage renal disease (Rossing, 2006). Increased thickness of glomerular basement membrane and augmentation of glomerular extracellular matrix (ECM) are recognized as pathological hallmarks of diabetic nephropathy (Hisashi *et al.*, 2006). Thus, glomerular injury is apparently critical for the initiation and progression of the disease. Upregulation of transforming growth factor-β 1 (TGF-β1) and connective tissue growth factor (CTGF) play an important role in the pathogenesis of diabetic nephropathy because they stimulate ECM accumulation and fibrotic effects (Guhua *et al.*, 2004). Four main hypotheses about how hyperglycaemia causes diabetic complications are: increased polyol pathway flux; increased advanced glycation end product (AGE) formation; activation of protein kinase C; increased hexosamine pathway flux. All seem to reflect a single hyperglycaemia-induced overproduction of reactive oxygen species (ROS) by the mitochondrial electron transport chain in endothelial cells (Brownlee, 2001; Nishikawa and Araki, 2007). Previous studies in type 2 diabetes have generally found an impairment in endothelium-dependent vasodilatation; endothelium-independent dilatation, however, has been variably reported to be reduced or unaffected (Natali *et al.*, 2006).

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Haemodynamic factors are also implicated in the pathogenesis of diabetic nephropathy and include elevations of systemic and intraglomerular pressure and activation of various vasoactive hormone pathways including the renin–angiotensin aldosterone system, endothelin and urotensin (Forbes *et al*., 2007).

Urocortin was first described by Vaughan *et al*. (1995) as a 40-amino-acid peptide related to the corticotrophin-releasing factor (CRF) family and it binds to and activates both type 1 and type 2 CRF receptors (Vaughan *et al*., 1995; Donaldson *et al*., 1996). Urocortin is distributed both in the CNS and periphery, in sites such as the Edinger–Westphal nucleus, adipose tissue, heart, kidney and immunological tissue (Fekete and Zorrilla, 2007). Endothelial urocortin suppressed the generation of angiotensin II-induced ROS production in endothelial cells (Honjo *et al*., 2006). The urocortin-induced endothelium-dependent relaxation of rat artery has been reported (Huang *et al*., 2002). This peptide is found in the kidney (Kageyama *et al*., 1999) and produces a marked vasodilatation of renal arteries, which is reduced in diabetic animals (Sanz *et al*., 2003). Four-day administration of urocortin has sustained beneficial haemodynamic, hormonal, and renal effects in experimental heart failure (Rademaker *et al*., 2005). We have previously demonstrated that urocortin may play a protective role in ischemia-reperfusion injury in rat hearts against oxidative stress by inhibiting the activities of free radicals (Liu *et al*., 2005). Urocortin is also found to have an inhibitory effect on the activity of serum angiotensin-converting enzyme (Yang *et al*., 2006). Taken together, these reports strongly suggest that urocortin might have a beneficial influence on diabetic nephropathy.

To the best of our knowledge, there are still no reports on the relationship between urocortin and diabetic nephropathy. We hypothesized that diabetic nephropathy could be reversed by urocortin. In the present study, we have examined the effects of urocortin on diabetic nephropathy, as expressed by characteristics such as ECM expansion, AGE formation, and sorbitol accumulation, in *db/db* mice, a genetic model of type 2 diabetes with obesity and insulin resistance. Diabetic nephropathy in this strain exhibits characteristic changes resembling those found in human diabetic nephropathy (Like *et al*., 1972). We also investigated the *in vitro* effects of urocortin on the overexpression of TGF-β1 and CTGF induced by exposing mesangial cells to high glucose media. These cells play a pivotal role in the ECM expansion and accumulation in diabetic nephropathy.

Methods

Animals

All animal procedures and experiments were conducted in accordance with the official recommendations of the Chinese Community Guidelines. Male and female Lepr^{db} (*db/db*) mice were purchased from Mutant Mice Model Animal Research Center (MARC) at ~6 weeks of age and were housed in the Nanjing University of Chinese Medicine Animal Resource Center.

Experimental protocols

At 9 weeks of age, after onset of hyperglycaemia which occurred at ~5 weeks after birth, mice were divided into three groups (eight animals per group): normal saline group, urocortin-treated group and urocortin + astressin group. Mice in the urocortin group received 7 μg kg^{-1} urocortin and mice in the urocortin + astressin group received 7 + 35 μg kg^{-1} astressin intraperitoneally, daily for 6 weeks. Mice in the normal saline group received the same amount of normal saline. Age matched mice with the same genetic background (C57BLKS/J) were used as controls (eight animals). Control (*db/m*) and *db/db* mice were housed four per cage in a room with a 12 h artificial light cycle and free access to standard diet and water. Food intake was measured daily and body weight was weighed weekly. Blood samples from the tail vein were used to determine the non-fasting blood glucose with blood glucose test strips (Roche diagnostics GmbH, D-68298 Mannheim, Germany) weekly.

At the end of the experiment, mice were anaesthetized (ketamine/xylazine; 70/10 mg kg^{-1} i.p.) and blood samples were withdrawn from the retrobulbar venous plexus with a capillary and anticoagulated with heparin (25 units). Plasma was frozen at –80 °C for biochemical and RIA analysis. Red blood cells (RBC) were washed with normal saline and a 20%(v/v) RBC suspension was prepared with double-distilled water, frozen and thawed three times and stored at −20 °C for sorbitol detection. Kidneys were removed, decapsulated and weighed. One kidney from each mouse was frozen at –80 °C until it was used to provide a homogenate; the other was removed for histological examination.

Biochemical and RIA analysis

Plasma glucose and blood urea nitrogen (BUN) were measured using the commercially available kits (Nanjing Jiancheng Bioengineering Institute). Plasma insulin was measured by RIA (Beijing Atom High Tech Co. Ltd). Serum AGE levels were determined as described previously (Munch *et al*., 1997). In brief, serum samples were diluted 100-fold in PBS and filtered (0.22 μm, Millex-GV; Millipore, Bedford, MA, USA). Haemolytic sera were excluded from AGE measurements. Fluorescence spectra (corrected for background) were recorded in a Microplate Fluorometer (GEMINI, Molecular Device, USA) at room temperature. The excitation and emission wavelengths were set to 370 and 445 nm, respectively and AGE concentration was expressed in fluorescent units.

Measurement of RBC sorbitol concentration

The RBC suspension was thawed out and absolute ethanol was used to precipitate protein and then centrifuged at 1200 × *g* for 15 min. The supernatant was used to determine sorbitol concentration by a modified fluorometric enzyme assay (Gupta *et al*., 2002). The final reaction mixture consisted of 0.2 M glycine-NaOH buffer, pH 9.4, and 0.3 mM NAD^+, with or without 4U sorbitol dehydrogenase. Samples were incubated in dark with shaking at 37 °C for 30 min. Sample fluorescence was determined at a wavelength of 460 nm with excitation at 338 nm with Microplate Fluorometer (GEMINI,

Molecular Device, USA). Sorbitol content was calculated by comparison with appropriate standards and blanks.

Preparation of tissue homogenate and measurement of SOD activity and MDA level

Kidney homogenate (10%, w/v) was prepared with 0.1 M PBS and centrifuged at $1200 \times g$ for 10 min. The supernatant was used to determine SOD activity and MDA levels with commercially available kits (Nanjing Jiancheng Bioengineering Institute).

Histological analysis

Kidneys were fixed in 10% formalin for 24 hours. Sections (2 μm) were stained with periodic acid Schiff (PAS) and haematoxylin–eosin (H&E) stain for light microscopic examination. The severity of renal pathological changes was scored by plural analysis on an arbitrary 0–4-point scale from light to serious. The degree of glomerular injury (glomerulosclerosis) was evaluated by a semi-quantitative method as described previously (Bilous *et al*., 1989). In brief, kidney sections stained with PAS were observed under a light microscope in a masked fashion at a magnification of $\times 400$. Both total glomerular area (including nuclei and spaces within capillary loops) and mesangial area (excluding nuclei) were obtained by using an Olympus microscope and JVC camera. For each glomerulus, the ratio of mesangial area to total glomerular area was calculated by using the Analysis Imaging System (OPTMAS, Imaging Research, St Catherine's, ON, Canada). We recorded and assessed $\geqslant 20$ glomeruli from each mouse. The result ('per cent mesangial area') was used to determine the severity of glomerulosclerosis in each animal (Lenz *et al*., 1998).

Cell culture

Rat mesangial cells (MC) (HBZY1) were purchased from the China Center for Type Culture Collection. This cell line was originated from Sprague–Dawley rats. MC were maintained at 37 °C in a humidified incubator with 5% CO_2/95% air and propagated in low glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% newborn bovine serum.

Sterilized poly-L-lysine coated cover slips were placed in 6-well plates and 1×10^5 cells were seeded in 2 mL medium. Serum-free media containing 25 mM glucose or 5 mM glucose was changed 24 h after seeding and cultured with various testing reagents for 4 days. The cells were divided into four groups: 5 mM glucose group, 25 mM glucose group, 25 mM glucose with 10^{-9} M urocortin group and 25 mM glucose with 10^{-9} M urocortin $+ 5 \times 10^{-9}$ M astressin group. Every group was replicated three times in different 6-well plates. At the end of the fourth day, the media was removed and cover slips were rinsed with PBS for immunocytochemical assays.

Immunocytochemical assay

Cover slips with adherent MC were washed with PBS and fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100. Coverslips were rinsed in PBS. A modification of the ABC immunoglobulin enzyme bridge technique was used for immunocytochemistry according to the manufacturer's instruction. In brief, primary antibodies were (rabbit anti-CTGF and goat anti TGF-β1) added and incubated for 24 h at 4 °C. Coverslips were rinsed in PBS. Secondary antibody was added and incubated for 30 min with gentle rocking. Substrate (DAB) solution was prepared before use and added to the coverslips followed by incubation in the dark for about 20 min. Colour reaction was stopped by blotting off substrate and rinsing in water for 5–10 min.

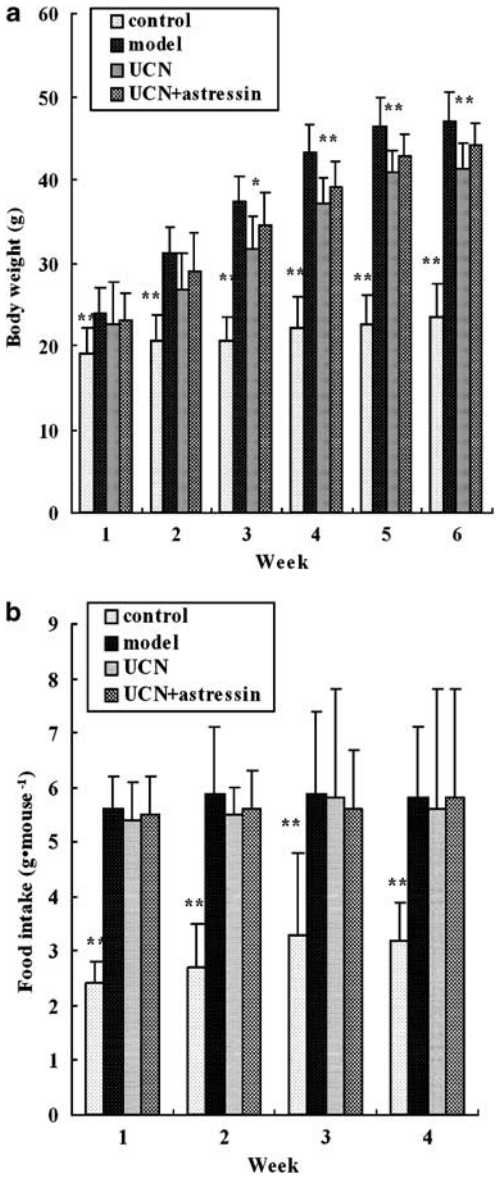

Figure 1 Effects of urocortin on body weight and food intake in *db/db* mice. (**a**) Body weight of *db/db* mice was decreased by urocortin ($7\ \mu g\ kg^{-1}$, ip). Pretreatment with astressin ($35\ \mu g\ kg^{-1}$, ip) prevented the effect of urocortin. (**b**) Food intake was not affected either by treatment with urocortin or by urocortin + astressin. Control, nondiabetic control; model, *db/db* mice = diabetic control; urocortin, diabetic mice (*db/db* mice) treated with urocortin ($7\ \mu g\ kg^{-1}$, ip); urocortin + astressin, diabetic mice (*db/db*) treated with urocortin and (7 and $35\ \mu g\ kg^{-1}$ respectively, ip). $^*P<0.05$, $^{**}P<0.01$ compared with model.

Data analysis and statistical procedures

Data were presented as means ± s.e.m. ANOVA was used for comparison followed by a *post hoc* test for between-group comparison. Differences were considered significant if $P<0.05$.

Materials

Urocortin, astressin and sorbitol dehydrogenase were purchased from Sigma; primary antibodies (rabbit anti-CTGF and goat anti TGF-β1) were purchased from Santa Cruz; Secondary antibodies were purchased from JINGMEI BIOTECH; DAB was purchased from GeneTech.

Results

Effects of urocortin on body weight and food intake

Body weight of *db/db* mice is reported to increase significantly at the onset of hyperglycaemia and reach to a peak by 1 month after hyperglycaemia (approximately twofold increase over age-matched controls), then remain constant over the next 2 weeks (Starkey *et al*., 2006). We divided *db/db* mice at the age of 9 weeks when hyperglycaemia state and body weight were stable, and found no difference in blood glucose among the groups of *db/db* mice (data not shown). Body weight of *db/db* mice began to decrease at the third week of urocortin treatment and was maintained for the next 3 weeks, however, the CRF receptor blocker, astressin, appeared to antagonize the effect of urocortin on body weight (Figure 1a). Food intake was not affected either by urocortin or urocortin + astressin treatment (Figure 1b). The food intake was measured by using the average intake of four mice in each cage.

Effects of urocortin on plasma insulin, blood glucose, BUN, creatinine, AGE level and RBC sorbitol concentration

As shown in Figures 2a and b, the high plasma insulin and glucose level of *db/db* mice (about 3–4-fold increased over age-matched controls) were not affected by treatment with urocortin (Figure 2a shows the blood glucose value at the end of the experiment. Weekly values are not shown). Compared with age-matched *db/m* mice, plasma BUN and creatinine levels of *db/db* mice were clearly increased and this increase was diminished by treatment with urocortin (Figures 2c and d). This effect of urocortin on BUN and creatinine was significantly affected by astressin.

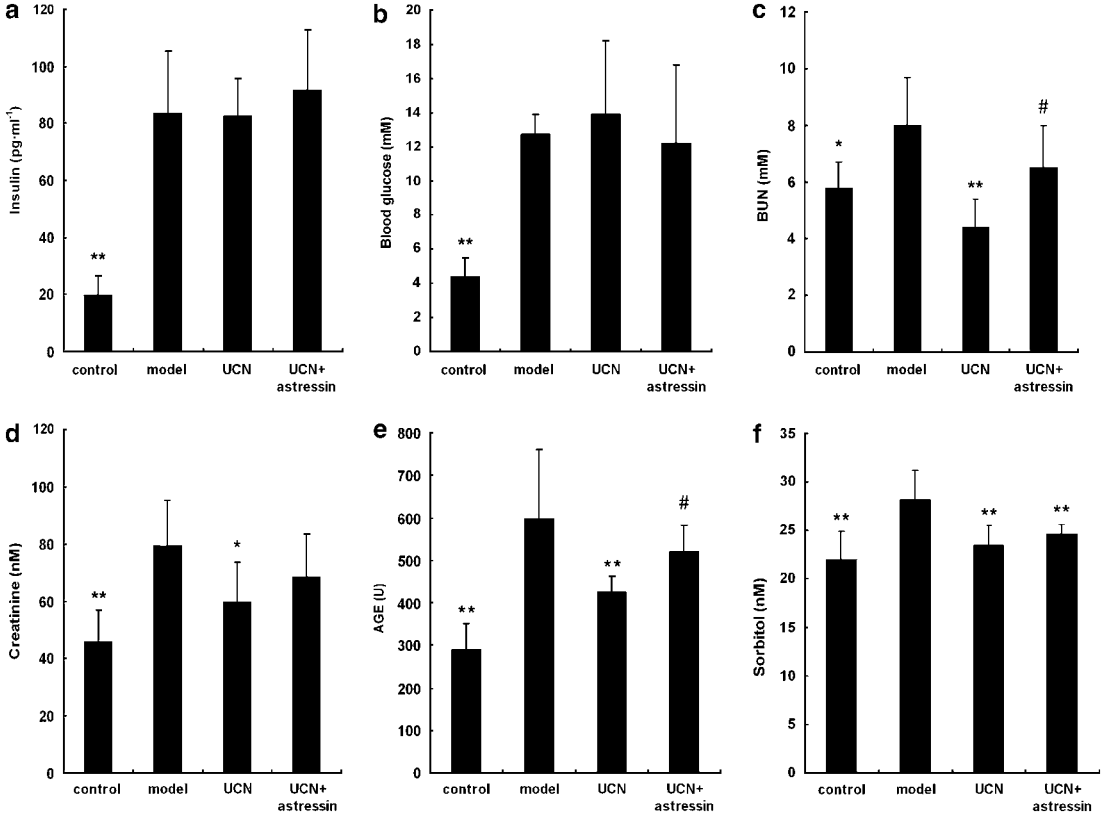

Figure 2 Effects of urocortin on plasma insulin, blood glucose, BUN, creatinine, AGE level and RBC sorbitol concentrations in *db/db* mice. Plasma insulin level (**a**) and blood glucose level (**b**) were not affected by urocortin (7 μg kg^{-1}, ip) and urocortin (7 μg kg^{-1}, ip) + astressin (35 μg kg^{-1}, ip); Plasma BUN (**c**) and creatinine (**d**) level of *db/db* mice were decreased by urocortin; Plasma AGE level (**e**) and RBC sorbitol concentration (**f**) were decreased by urocortin. Pretreatment with astressin abolished the effect of urocortin on AGE, BUN and creatinine but not its effect on sorbitol. Control, nondiabetic control; model, *db/db* mice = diabetic control; urocortin, diabetic mice (*db/db* mice) treated with urocortin (7 μg kg^{-1}, ip); urocortin + astressin, diabetic mice (*db/db*) treated with urocortin and (7 and 35 μg kg^{-1} respectively, ip).*$P<0.05$, **$P<0.01$ compared with model; $^{\#}P<0.05$ compared with urocortin.

In diabetes, prolonged hyperglycaemia drives the glycation reaction and non-enzymatic crosslinking between proteins and glucose or its derivatives. A series of further complex molecular rearrangements yield irreversible AGE (Brownlee *et al*., 1988). In the present study, the high plasma levels of AGE in *db/db* mice (about twofold increased over age-matched *db/m* mice) was decreased by treatment with urocortin and this effect was abolished by pretreatment with astressin (Figure 2e).

In addition, hyperglycaemia also activates the polyol pathway and sorbitol levels of RBC are recognized as an indicator of activity of the polyol pathway (Malone *et al*., 1984). As demonstrated in Figure 2f, RBC sorbitol concentration was much higher in *db/db* mice than that in *db/m* mice and was decreased after treatment with urocortin. Pretreatment with astressin did not influence the effect of urocortin on RBC sorbitol concentration.

UCN-induced changes in SOD activity and MDA level in kidney homogenates

As shown in Figure 3, SOD activity was lower and MDA level higher in the kidney of *db/db* mice compared with *db/m* mice. Urocortin significantly enhanced SOD activity and reduced MDA levels in kidney homogenates. These effects of urocortin could be prevented by pretreatment with astressin.

Inhibition of glomerular ECM expansion and accumulation by urocortin

Diabetic nephropathy is characterized by an expansion of glomerular mesangium, which is caused by mesangial cell proliferation and excessive accumulation of ECM (Ahn *et al*., 2004). As demonstrated in Figure 4, in the *db/m* mice, the outer cortical glomerulus was of normal size and configuration. Moreover, the mesangium contained the usual complement of cells and matrix without ECM accumulation. The glomeruli from *db/db* mouse kidneys were dramatically different in appearance. The glomeruli had a severe degree of mesangial matrix expansion and the mesangium was diffusely and markedly expanded with PAS-positive matrix material. Mesangial matrix expansion and accumulation of PAS-positive matrix material (per cent mesangial area) were prevented by urocortin. Although astressin treatment did not result in statistically significant reversal of urocortin effect, the degree of ECM expansion and accumulation was more severe.

UCN-induced reduction in TGF-β1 and CTGF expression in mesangial cells

High glucose stimulates expression of TGF-β1 and CTGF in MC (Murphy *et al*., 1999; Mclennan *et al*., 2004). An earlier study (Mclennan *et al*., 2004) and our own results (data not shown) showed that this stimulatory effect of 25 mM glucose could not be ascribed to changes in the osmotic state of culture media, as the same amount of mannitol was used to provide an osmotic control. Expression of TGF-β1 and CTGF was increased significantly when MC were cultured in 25 mM glucose DMEM compared with 5 mM glucose and these increases were reversed by incubation with urocortin. Pretreatment with astressin completely abolished the inhibitory effect of urocortin on TGF-β1 and CTGF overexpression as shown in Figures 5 and 6.

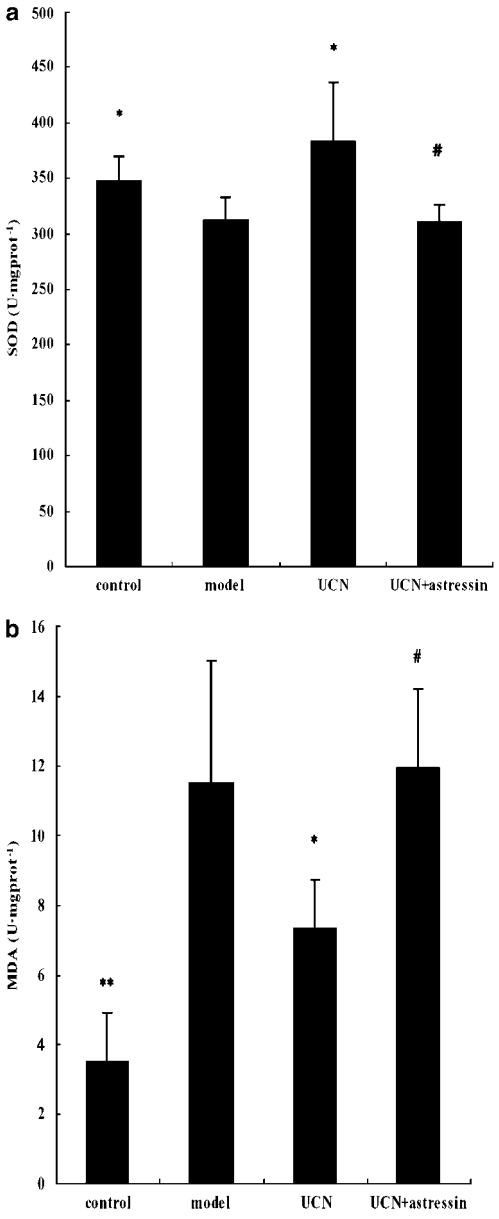

Figure 3 Urocortin-induced changes in SOD activity and MDA level in kidney homogenates of *db/db* mice. SOD activity (**a**) was increased and MDA level (**b**) was decreased by urocortin. Astressin abolished the effect of urocortin on SOD activity and MDA level. Control, nondiabetic control; model, *db/db* mice = diabetic control; urocortin, diabetic mice (*db/db* mice) treated with urocortin (7 μg kg^{-1}, ip); urocortin + astressin, diabetic mice (*db/db*) treated with urocortin and (7 and 35 μg kg^{-1} respectively, ip). *$P<0.05$, **$P<0.01$ compared with model; $^{\#}P<0.05$ compared with urocortin.

These results indicated that the inhibitory effect of urocortin on ECM expansion and accumulation was closely related to its influence on expression of TGF-β1 and CTGF, which play pivotal roles in ECM generation and degradation.

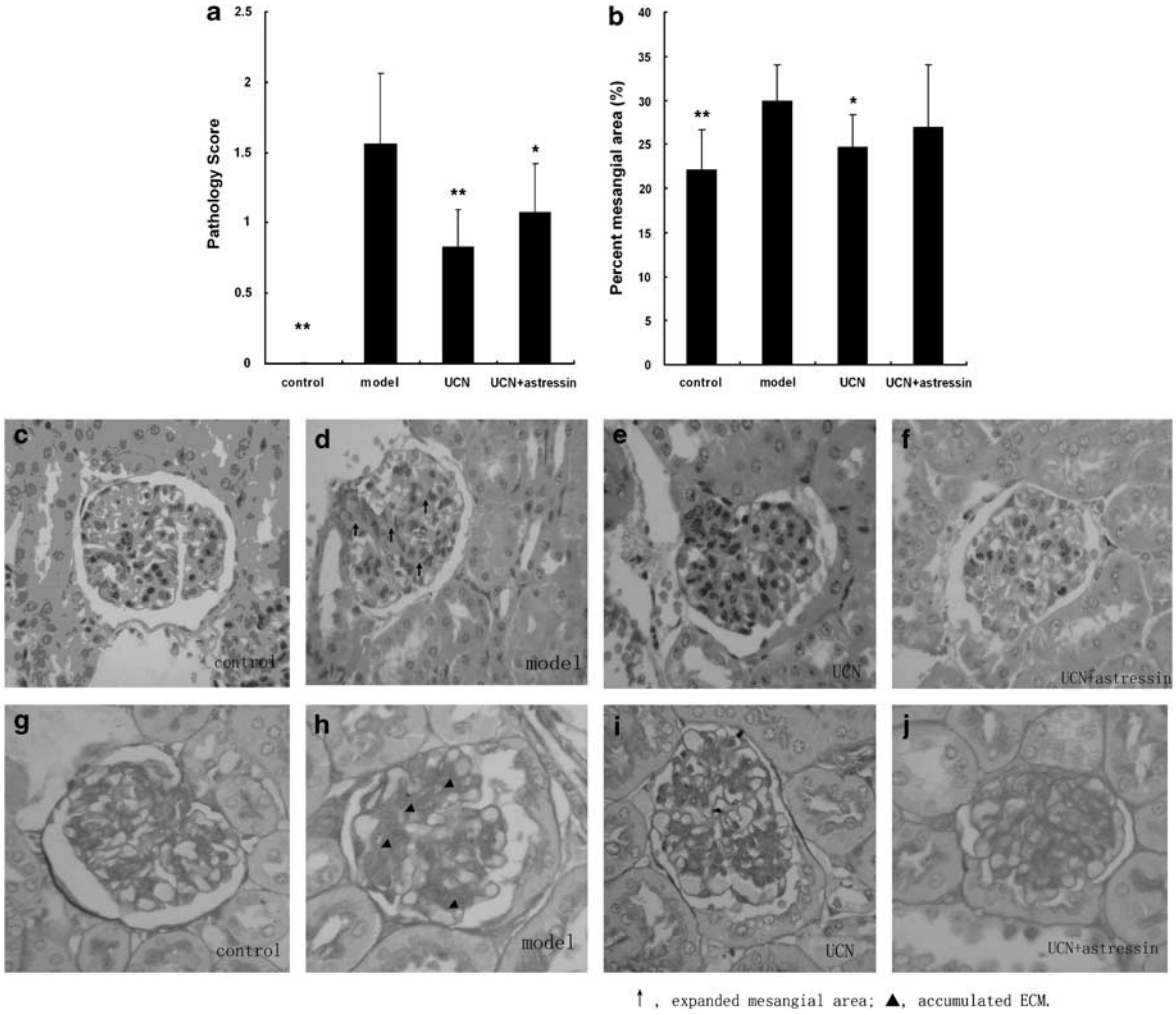

Figure 4 Protective effect of urocortin on glomerular histology in *db/db* mice. (**a**) Pathology score; (**b**) Per cent mesangial area; Representative micrographs of H&E-stained paraffin sections showed effect of urocortin on glomerular histology in *db/db* mice (**c–f**; magnification $\times 400$). (**c**) Glomeruli of *db/m* mice; (**d**) Glomeruli of *db/db* mice; (**e**) Glomeruli of *db/db* mcie treated with urocortin ($7\,\mu g\,kg^{-1}$); (**f**) Glomeruli of *db/db* mice treated with urocortin ($7\,\mu g\,kg^{-1}$) + astressin ($35\,\mu g\,kg^{-1}$); Representative micrographs of PAS-stained paraffin sections showed effect of urocortin on ECM expansion and accumulation (**g–j**; magnification $\times 400$). (**g**) Glomeruli of nondiabetic control mice; (**h**) Glomeruli of *db/db* mice; (**i**) Glomeruli of *db/db* mice treated with urocortin ($7\,\mu g\,kg^{-1}$); (**j**) Glomeruli of *db/db* mice treated with urocortin ($7\,\mu g\,kg^{-1}$) + astressin ($35\,\mu g\,kg^{-1}$). Treatment with urocortin clearly decreased the ECM expansion and accumulation. Although astressin treatment did not result in statistically significant reversal of the effect of urocortin, the degree of ECM expansion and accumulation was more severe. Control, nondiabetic control; model, *db/db* mice = diabetic control; urocortin, diabetic mice (*db/db* mice) treated with urocortin ($7\,\mu g\,kg^{-1}$, ip); urocortin + astressin, diabetic mice (*db/db*) treated with urocortin and (7 and $35\,\mu g\,kg^{-1}$ respectively, ip). *$P<0.05$; **$P<0.01$ compared with model. △, expanded mesangial area; ▲, accumulated ECM.

Discussion and conclusions

In the current study, we examined the effect of urocortin on type 2 diabetic nephropathy, whose characteristics are expressed by changes in AGE formation, polyol pathway activation, ECM accumulation and ROS production.

Reducing sugars (including glucose, fructose, and trioses) can react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and then Amadori products. These early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to become irreversibly crosslinked heterogeneous fluorescent derivatives, termed AGE. The formation and accumulation of AGE mediates the progressive alteration in renal architecture and loss of renal function (Yamagishi *et al*., 2002) and is considered to be important for the progression of diabetic nephropathy (Sugimoto *et al*., 2007). Increased polyol pathway activity induced by hyperglycaemia has been reported to contribute to abnormalities such as increased osmotic and oxidative stress factors that have been cited as promoters of diabetic microvascular diseases including diabetic nephropathy (Dunlop, 2000). High plasma MDA level and decreased SOD activity are found in obese patients with type 2 diabetes (Mohora *et al*., 2006). Antioxidant vitamins (for example, vitamins C and E) protect against the development of diabetic nephropathy (Kedziora-Kornatowska *et al*., 2003). In the present study, urocortin was found to decrease the accumulation of AGE and the activity of the

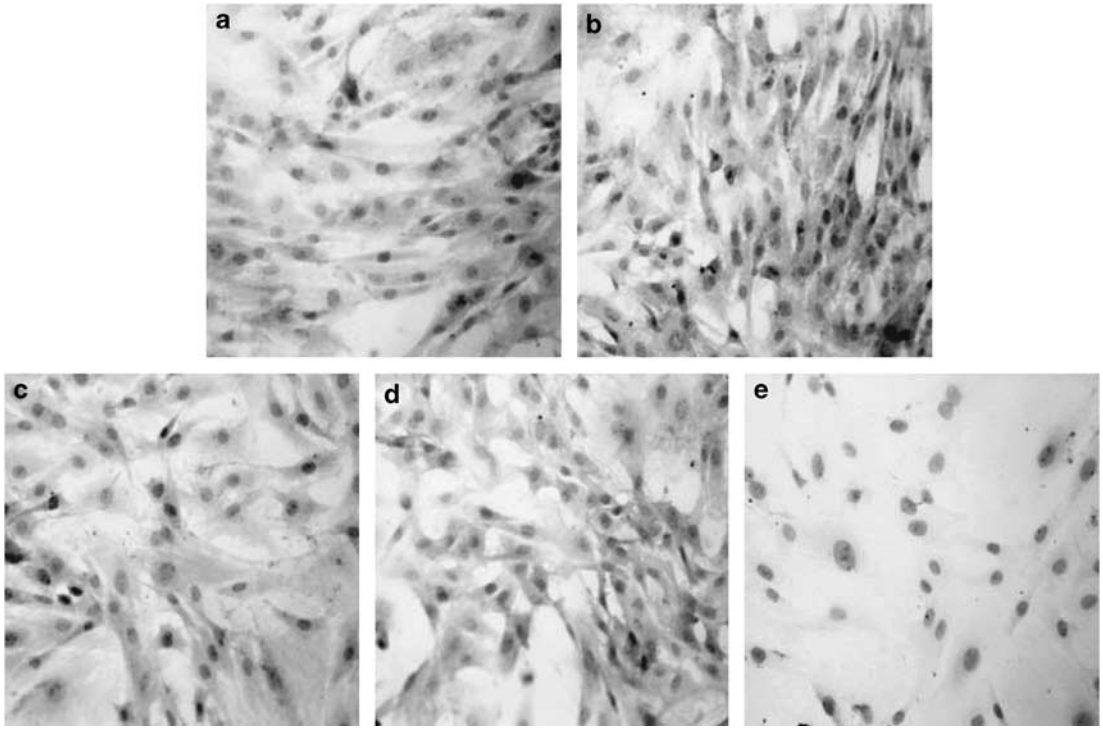

Figure 5 Urocortin-induced reduction in transforming growth factor-β1 (TGF-β1) expression in mesangial cells (MC). Expression of TGF-β1 was detected by immunocytochemistry (magnification, $\times 200$). MC were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose or 25 mM glucose with urocortin (10^{-9} M) or urocortin (10^{-9} M) + astressin (5×10^{-9} M). (**a**) Normal DMEM; (**b**) DMEM contained 25 mM glucose; (**c**) DMEM contained 25 mM glucose and urocortin; (**d**) DMEM contained 25 mM glucose and urocortin + astressin; (**e**) Negative control. Overexpression of TGF-β1 was inhibited by urocortin and pretreatment with astressin abolished this effect.

polyol pathway. AGE-modified macromolecules are susceptible to elimination via the scavenger receptor of Kuppffer cells (Smedsrod *et al*., 1997) and urocortin and its CRF receptors are expressed in rat Kupffer cells (Charalampopoulos *et al*., 2006). Our results showed that pretreatment with the CRF receptor antagonist, astressin, prevented the effect of urocortin on AGE. This new finding may imply that urocortin might participate in AGE elimination via CRF receptors in Kupffer cells. Although there are some interactions between AGE and the polyol pathway (Dan *et al*., 2004), the relationship between them is not clear. Whether the inhibitory effect of urocortin on the activity of the polyol pathway was a direct effect or was related to AGE is a question that needs further investigation, as this effect was not affected by astressin. In accordance with our previous results (Liu *et al*., 2005), urocortin decreased MDA level and increased SOD activity in kidney homogenate and this antioxidative effect was closely related to CRF receptors.

BUN and creatinine levels are higher in rats with diabetic nephropathy than those in normal rats (Sun *et al*., 2006). Increased thickness of glomerular basement membrane and augmentation of glomerular ECM are recognized as pathological hallmarks of diabetic nephropathy. In response to high level of glucoses, the output of the potent profibrotic factor, TGF-β1, significantly increases, which leads to fibrotic consequences (Park *et al*., 1997). In the development of ECM accumulation, CTGF may act as a downstream mediator of TGF-β1 (Umezono *et al*., 2006). Our results showed that the high plasma BUN and creatinine level and the augmentation of glomerular ECM were reduced by urocortin. To elucidate the molecular mechanism of the protective effects of urocortin on diabetic nephropathy, we did experiments *in vitro*, using MC to assess the role of urocortin in the expression of the two important factors, TGF-β1 and CTGF. Another new finding from our experiments was that urocortin could inhibit overexpression of TGF-β1 and CTGF induced by high glucose in MC cultures. These *in vitro* results indicated that the inhibition by urocortin of glomerular ECM augmentation and accumulation *in vivo* were likely to be mediated by its inhibitory effect on overexpression of TGF-β1 and CTGF by MC.

Previous reports show that peripheral administration of urocortin decreases food intake and increases energy expenditure (Hope *et al*., 2000; Kinney *et al*., 2000). Decreased food intake induced by urocortin occurs when this peptide urocortin binds to leptin, which increases leptin's entry into brain and thus reducing appetite. However, this effect of the bound complex needs the help of leptin receptors at the blood-endothelial cell interface of blood–brain barrier (Kastin *et al*., 2002). Our results showed that urocortin injection did not affect food intake of *db/db* mice, which is at variance with previous reports. This discrepancy may be due to the deficiency in leptin receptors in *db/db* mice (Sharma *et al*., 2003). Leptin has been reported to play a role in the development of diabetic nephropathy (Fruehwald-Schultes *et al*., 1999). However, some researchers found that leptin

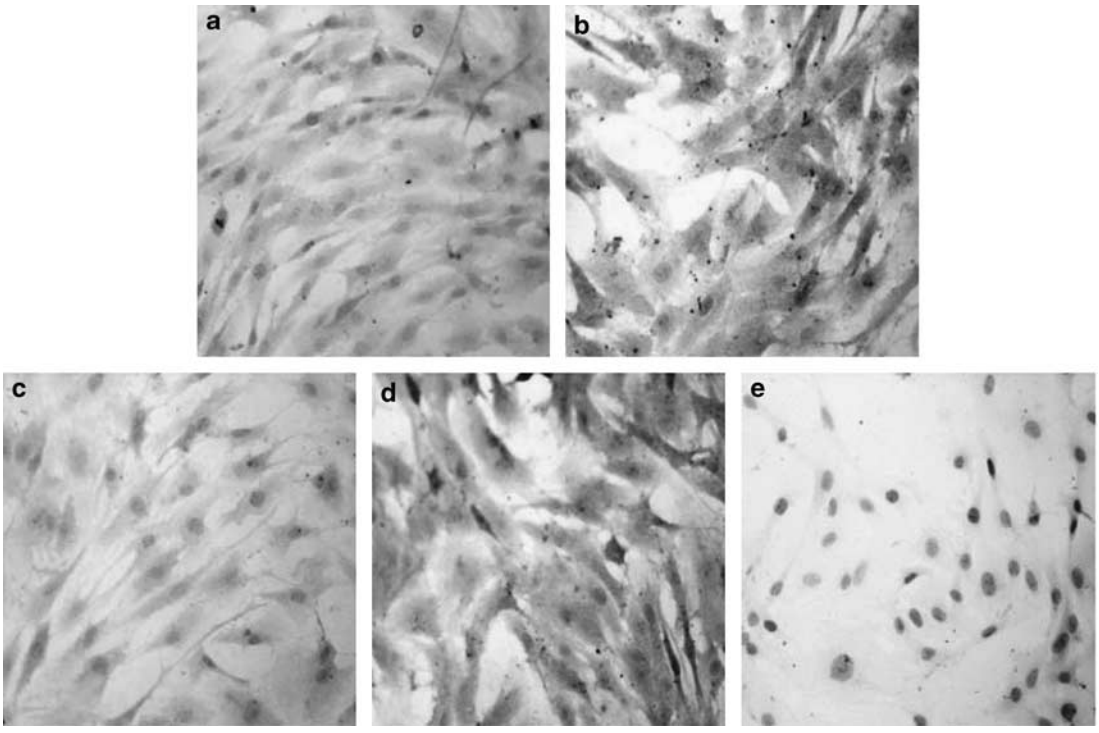

Figure 6 Urocortin-induced reduction in connective tissue growth factor (CTGF) expression in rat mesangial cells (MC). Expression of CTGF was detected by immunocytochemistry (magnification, $\times 200$). MC were cultured in serum-free DMEM containing 5 mM glucose or 25 mM glucose with urocortin (10^{-9} M) or urocortin (10^{-9} M) + astressin (5×10^{-9} M). (**a**) Normal DMEM. (**b**) DMEM contained 25 mM glucose. (**c**) DMEM containing 25 mM glucose and urocortin. (**d**) DMEM containing 25 mM glucose and urocortin + astressin. (**e**) Negative control. The overexpression of CTGF stimulated by the high glucose medium was inhibited by urocortin and pretreatment with astressin reversed this effect.

could be used for the treatment of lipodystrophy and hence is beneficial for diabetic nephropathy (Javor *et al*., 2004). This discrepancy is difficult to explain. Although urocortin was not observed here to affect food intake, it was found to reduce the body weight of *db/db* mice. This decreasing effect may be attributed to other factors, such as fluid retention and energy expenditure. Body weight is a relatively variable index; for example, it can be affected by food or water intake. Moreover, the status of diabetic nephropathy also contributes to body weight because of the different degrees of water retention.

In addition, our results showed that urocortin (UCN 1) injection did not affect blood glucose level and secretion of insulin in *db/db* mice. Previous reports show that urocortin may play a role in regulation of glucose, glucagon and insulin level. For example, another form UCN 2 has a function as a local negative regulator of glucose uptake and insulin sensitivity in skeletal muscle (Chen *et al*., 2006). UCN-3 is expressed in pancreatic β cells and injections of this peptide result in an increase in plasma insulin levels and an elevation of plasma glucagon followed by reduced plasma glucose levels *in vivo* (Li *et al*., 2003). This inconsistency may be due to the different types of urocortin used for investigation.

In conclusion, urocortin ameliorated diabetic nephropathy. Inhibition of AGE accumulation, polyol pathway activation, ECM expansion and accumulation, TGF-β1 & CTGF overexpression and ROS overproduction may all contribute to the amelioration of diabetic nephropathy by urocortin.

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Conflict of interest

The authors state no conflict of interest.

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