Gliquidone decreases urinary protein by promoting tubular reabsorption in diabetic Goto-Kakizaki rats

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Abstract

The efficacy of gliquidone for the treatment of diabetic nephropathy was investigated by implanting micro-osmotic pumps containing gliquidone into the abdominal cavities of Goto-Kakizaki (GK) rats with diabetic nephropathy. Blood glucose, 24 h urinary protein, and 24 h urinary albumin levels were measured weekly. After 4 weeks of gliquidone therapy, pathological changes in the glomerular basement membrane (GBM) were examined using an electron microscope. Real-time PCR, western blotting, and immunohistochemistry were employed to detect glomerular expression of receptors for advanced glycation end products (RAGE) (AGER), protein kinase C β (PKC β), and protein kinase A (PKA) as well as tubular expression of the albumin reabsorption-associated proteins: megalin and cubilin. Human proximal tubular epithelial cells (HK-2 cells) were used to analyze the effects of gliquidone and advanced glycation end products (AGEs) on the expression of megalin and cubilin and on the absorption of albumin. Gliquidone lowered blood glucose, 24 h urinary protein, and 24 h urinary albumin levels in GK rats with diabetic nephropathy. The level of plasma C-peptide increased markedly and GBM and podocyte lesions improved dramatically after gliquidone treatment. Glomerular expression of RAGE and PKC β decreased after gliquidone treatment, while PKA expression increased. AGEs markedly suppressed the expression of megalin and cubilin and the absorption of albumin in HK-2 cells *in vitro*, whereas the expression of megalin and cubilin and the absorption of albumin were all increased in these cells after gliquidone treatment. In conclusion, gliquidone treatment effectively reduced urinary protein in GK rats with diabetic nephropathy by improving glomerular lesions and promoting tubular reabsorption.

Key Words

- ▶ gliquidone
- ▶ diabetic nephropathy
- ▶ proteinuria
- ▶ mechanism

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Introduction

Diabetic nephropathy is one of the most common, chronic, complications of diabetes mellitus (DM) and is a major contributing factor in end-stage kidney

pathology. The major pathogenic mechanism of diabetic nephropathy is the occurrence of glomerular microvascular lesions, though the underlying mechanism

responsible for the development of these lesions remains elusive.

Advanced glycation end products (AGEs) have been found to be a major cause of diabetic microvascular lesions. AGEs may act upon the glomerular capillary receptor of AGE (RAGE), activate protein kinase C (PKC), initiate oxidation stress reactions and/or cause a large accumulation of – reactive oxygen species (ROS) and nitric oxide, all of which can injure the glomerular microvascular endothelial cells (Brownlee 2001, Kanwar *et al.* 2008). AGEs can also increase extracellular matrix and mesangial cell proliferation (Ziyadeh 1993). The diabetic glomerular lesions include structural and functional changes in glomerular filtration membrane barriers, such as thickening of the glomerular basement membrane (GBM) and loss of podocytes (Bangstad *et al.* 1994, Wolf *et al.* 2005), resulting in increased protein excretion. In fact, tubular lesions have been found in diabetics even when the urinary albumin excretion rate is within a normal range. It has been reported that tubular lesions and renal interstitial fibrosis are more closely correlated to renal function than is glomerular sclerosis (Magri & Fava 2009). AGE conjugates with RAGE to activate PKC and increases the production of cytokines (Kanwar *et al.* 2008). They also alter the expression of the reabsorption-associated proteins, megalin and cubilin, on the membrane surfaces of epithelial cells and affect the reabsorption of urinary protein (Tojo *et al.* 2003, Amsellem *et al.* 2010). Glomerular vascular lesions lead to increased protein leakage and tubular lesions result in decreased protein reabsorption, therefore these lesions are the major pathophysiological cause for the occurrence of proteinuria in diabetic nephropathy (Tryggevason *et al.* 2006, Russo *et al.* 2009).

PKC activation in glomerular capillaries has been considered to be the most important link during the pathogenesis of diabetic nephropathy, and there is experimental evidence that PKC inhibitors could effectively prevent and control diabetic nephropathy (Tuttle *et al.* 2005). Recently, a correlation between the pathogenesis of diabetic nephropathy and decreased protein kinase A (PKA) activity in the glomerular capillary was identified. PKC activity is increased and PKA activity is decreased in diabetic nephropathy, thus the PKC/PKA balance is disturbed. Treatment with a PKA agonist may also effectively prevent and/or treat diabetic nephropathy (Wang *et al.* 2012).

Gliquidone is a second-generation hypoglycemic sulfonylurea that promotes the release of endogenous insulin from pancreatic β -cells and effectively lowers blood

glucose. It is rapidly absorbed by the intestinal tract when taken orally and has a biphasic stimulatory effect on insulin secretion. Approximately 95% of gliquidone is excreted through the bile and feces so drug accumulation is not a problem in patients with renal insufficiency. Therefore, the World Health Organization has recommended it as a first-line drug for the treatment of mild to moderate renal lesions in patients with diabetic nephropathy. In addition with lowering the level of plasma glucose, gliquidone could boost the sensitivities of peripheral tissues and hepatocytes to insulin (Malaisse 2006) and enhances the transcription activities of peroxisome proliferator-activated receptor γ (PPAR γ ; Lee *et al.* 2011). Yarat *et al.* (2001) demonstrated that gliquidone markedly lowered the levels of nonenzymatic glycosylation protein and total protein and increased the level of lens glutathione in diabetic rats. Yanardag *et al.* (2005) reported that gliquidone lessened the STZ-induced hepatic lesions by reducing oxidative stress in diabetic rats. In patients with diabetic nephropathy, substitution of oral glibenclamide with gliquidone has increased the glomerular filtration rate and lowered urinary albumin (Mazurov *et al.* 1998).

Although some studies have demonstrated the effectiveness of gliquidone in preventing and controlling diabetic nephropathy, the mechanism by which it improves diabetic nephropathy remains elusive. It could be that a higher plasma drug concentration enhances the effect of gliquidone or that gliquidone improves diabetic nephropathy by directly acting on the kidney, in addition to lowering glycemic levels. In this present study, a micro-osmotic pump that released gliquidone at a constant rate was used to treat diabetic nephropathy in Goto-Kakizaki (GK) rats. Pathological and functional changes in the kidneys were examined, both before and after treatment, and the effects of gliquidone on glomerular and tubular phenotype and function were analyzed in order to further elucidate the molecular mechanism by which gliquidone improves diabetic nephropathy.

Materials and methods

Animal model and groups

SPF-grade 8-week-old male GK rats, weighing about 250–300 g, were purchased from Shanghai Slac Laboratory Animal, Inc. (Shanghai, China). Rats were maintained on a 12 h light:12 h darkness cycle with free access to rodent chow and water. All animal experiments were performed in accordance with the protocols and guidelines approved

by the Animal Ethics Committee of China-Japan Friendship Hospital. After consumption of a high-fat diet for 2 weeks, blood glucose levels were determined in GK rats; rats that had blood glucose levels over 16.7 mM for 3 consecutive days were classified as diabetic. Diabetic rats were then fed conventional chow for an additional 10 weeks and 24 h urinary albumin was determined. When the 24 h urinary albumin level was >400 µg, the establishment of a diabetic nephropathy animal model was confirmed (Wang *et al.* 2012). A total of 18 GK rats with diabetic nephropathy were divided into three groups, according to a random number table ($n=6$ in each group), as follows. The gliquidone treatment group: an ALZET micro-amount osmotic pump, infused with 2 ml gliquidone solution, was surgically implanted into the abdominal cavity. The gliquidone solution was formulated with 99.8% dimethylformamide and the gliquidone solution was released at a constant rate of 40 nmol/kg per min. The insulin treatment group: insulin glargine 2.5 IU was administered once a day via s.c. injection. Diabetic nephropathy group: a micro-amount osmotic pump, infused with 2 ml of 99.8% dimethylformamide, was implanted into the abdominal cavity and the solution was released at the same rate as in the gliquidone treatment group. Six SPF-grade, male Wistar rats of similar age and body weight (250–300 g) were used as controls. The treatment duration was 4 weeks in all groups.

Detection of gliquidone concentration in the blood

To evaluate the effectiveness of drug delivery via micro-osmotic pump, gliquidone (10 and 50 mg/kg body weight per day) was administrated to rats through the micro-osmotic pump or by intragastric administration. HPLC with fluorescence detection was used for determination of gliquidone in rat serum. Stable drug concentrations were detected in the blood of all groups after 1 week of drug administration.

Detection of blood glucose, urinary protein, and urinary albumin

Blood glucose, urinary protein, and urinary albumin levels were detected at weeks 0, 1, 2, 3, and 4. Blood glucose levels were detected, via the tail vein, using a blood glucose meter (Johnson & Johnson, New Brunswick, NJ, USA). Animals were kept in metabolic cages for collection of 24 h urine samples. A BCA protein kit (Beyotime, NanTong, China) and rat albumin ELISA kit (Assaypro,

St Charles, MO, USA) were used to measure urinary protein and urinary albumin levels.

Detection of serum C-peptide

After 4 weeks of treatment, animals were killed with 2.5% pentobarbital sodium (i.p.) and serum was isolated from venous blood. A rat C-peptide RIA kit (R&D, Shanghai, China) was used to measure the concentration of C-peptide.

Electron microscopy

Rat renal tissues were fixed in 2.5% glutaraldehyde for 30 min at room temperature, rinsed with PBS and postfixed with 1% osmic acid. The specimens were then dehydrated and embedded into Epon812 to prepare slides for electron microscopy. GBM and podocyte morphologies were observed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan).

Isolation of glomerular and tubular tissues

After the 4-week treatment, both kidneys were surgically harvested from each rat after killing. The renal cortex was homogenized and passed through a 100-mesh sieve. The filtrate was applied to a 300-mesh metal sieve and the remaining glomeruli and filter tubules were harvested for subsequent experiments.

Human proximal tubular epithelial cell (HK-2 cell) culture

Human proximal tubular epithelial cells (HK-2) were maintained in DMEM/F12 culture medium (Sigma) containing 10% inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Digestion was done using 0.1% trypsin/0.1% EDTA.

HK-2 cells were cultured in medium containing AGEs (400 µg/ml, Abcam, Cambridge, UK), AGEs (400 µg/ml) + insulin (1 IU/ml), AGEs (400 µg/ml) + gliquidone (1.0 µg/ml, Double-crane Pharmaceuticals, Beijing, China), AGEs (400 µg/ml) + LY33531 (10 nM) or AGEs (400 µg/ml) + 8-Br-cAMP (100 µM) for either 24 (for real-time PCR) or 48 h (for albumin absorption and western blot). Albumin absorption and cubilin and megalin expression were evaluated as described below.

Evaluation of albumin absorption using HK-2 cells

The HK-2 cells were inoculated into a 96-well plate and cultured with AGEs, AGEs + insulin, AGEs + gliquidone,

AGEs+LY33531 or AGEs+8-Br-cAMP for 48 h. FITC-labeled human albumin (final concentration of 20 µg/ml, FITC-albumin, Abcam) was then added to the culture medium (Sidaway *et al.* 2004). After 30 min, the culture medium was removed and the cells were washed four times with PBS. The cellular fluorescence intensity of each well was then detected using a continuous spectrum fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). The protein concentration was analyzed by the BCA protein assay kit (Beyotime). The fluorescence intensity was corrected for cell numbers by total protein and expressed as fluorescence intensity per microgram of protein.

Real-time PCR

Total RNA was extracted using the SV total RNA isolation system (Promega) and 2 µg of total RNA was reverse transcribed using a reverse transcription kit (Promega). The SYBR green real-time PCR Master Mix (Toyobo, Shanghai, China) and ABI 7500 sequence detection system (Applied Biosystems) were employed for PCR amplification using the three-step method. Relative quantification of transcript levels was performed by the $2^{-\Delta\Delta C_t}$ method. The primers used for PCR amplification are shown in Supplementary Table 1, see section on supplementary data given at the end of this article (rat genes) and- Supplementary Table 2 (human genes).

Western blot

SDS-PAGE was used to fractionate 100 µg total protein and the protein was then transferred onto a PVDF membrane. Immunoblotting was performed using mouse anti-actin antibody (Sigma), mouse anti-RAGE antibody (Sigma), mouse anti-rat/human PKCβ antibody (Sigma), rabbit anti-mouse/human PKA antibody (Abcam), murine anti-rat megalin antibody (Abcam), goat anti-rat cubilin antibody (Santa Cruz Biotechnology, Inc.), rabbit anti-human megalin antibody (Abcam), and goat anti-human cubilin antibody (Santa Cruz Biotechnology, Inc.) respectively. ECL (Millipore, Shanghai, China) was used for detection.

Immunohistochemical staining

Rat pancreas tissue was fixed in 10% neutral formaldehyde and paraffin-embedded slides were prepared in order to evaluate insulin levels in the diabetic and gliquidone groups. The sections were immunostained with rabbit anti-rat insulin antibody and HRP-labeled secondary

antibody. After undergoing a reaction with DAB substrate and re-staining the nuclei, the slides were observed and photographed under a light microscope (Olympus, Tokyo, Japan).

Rat kidneys were fixed in 10% neutral formaldehyde and paraffin-embedded slides were prepared to evaluate the effect of gliquidone on protein reabsorption. The sections were immunostained with mouse anti-rat RAGE antibody, mouse anti-rat PKCβ antibody, rabbit anti-rat PKA antibody, mouse anti-rat megalin antibody, and goat anti-mouse cubilin antibody. Sections were then rinsed with PBS and HRP-labeled secondary antibody was added. After undergoing a reaction with DAB substrate and re-staining the nuclei, the slides were observed and photographed under a light microscope (Olympus).

Immunofluorescent staining of cells

HK-2 cells were inoculated into a 96-well culture plate and treated with AGEs (400 µg/ml) or AGEs (400 µg/ml) + gliquidone (1 µg/ml) for 48 h. The cells were then washed with PBS, fixed with 4% paraformaldehyde and blocked with 1% BSA/0.02% Tween. Immunostaining was done using mouse anti-human RAGE antibody, mouse anti-human PKCβ antibody, rabbit anti-human PKA antibody, rabbit anti-human megalin antibody, and goat anti-human cubilin antibody as the primary antibodies and Alexa fluor 488-conjugated secondary antibodies. The results were observed and photographed using a fluorescence microscope.

Statistical analyses

The data are expressed as mean±s.d. The software SPSS 17.0 was used for statistical analyses. A one-way ANOVA was used for intergroup data comparison. $P<0.05$ and $P<0.01$ denote statistical significance.

Results

Blood concentration of gliquidone after administration by micro-osmotic pump

One week after treatment, the average serum concentration of gliquidone in the group treated via pump administration was approximately five times higher than in the group treated via intragastric administration. This was true for both the 10 and 50 mg/kg groups. Furthermore, the gliquidone serum concentration was five times higher in the group that received 50 mg/kg

Figure 1

Concentration of gliquidone in the blood after administration by micro-osmotic pump. At the end of 1 week of treatment with gliquidone, serum was collected and the gliquidone concentration determined using HPLC with fluorescence detection. Intra-gastric: gliquidone was administered intra-gastrically to GK rats once daily at a dose similar to that received by rats in the micro-osmotic pump group. Osmotic pump: gliquidone was administered to GK rats via a micro-osmotic pump that was implanted into the abdominal cavity. 10 mg: drug dose is 10 mg/kg body weight; 50 mg: drug dose is 50 mg/kg body weight.

compared with the group that received 10 mg/kg gliquidone. Thus, a higher blood concentration of gliquidone was obtained via i.p. administration using a micro-osmotic pump (Fig. 1).

Effects of gliquidone on glycemia and proteinuria

Blood glucose levels decreased markedly during the treatment period in both the gliquidone and insulin groups (Fig. 2A). The 24 h urinary protein and urinary albumin levels increased in the diabetic nephropathy and insulin groups. However, in the gliquidone group, the 24 h urinary protein level (Fig. 2B) did not increase and the 24 h urinary albumin level (Fig. 2C) increased at a slower rate. After 2 weeks of treatment, there were significant differences in the 24 h urinary protein and albumin levels between the diabetic nephropathy and gliquidone groups ($P < 0.01$).

Effects of gliquidone on the secretion of insulin and C-peptide in rat islet cells

Insulin staining decreased markedly in the diabetic nephropathy group compared with the normal group. However, insulin staining in the gliquidone group was increased and approximated the normal level (Fig. 3A). Furthermore, the serum concentration of C-peptide was markedly lower in the diabetic nephropathy group than in the control group, but markedly higher in the gliquidone group than in the diabetic nephropathy group (Fig. 3B).

Effects of gliquidone on the morphologies of GBM and podocytes

The GBM in GK rats with diabetic nephropathy was obviously thickened and the foot processes of the podocytes were condensed or missing and in disarray compared with those of the normal controls. After 4 weeks of treatment with gliquidone, the GBM was markedly thinner and the morphologies and arrays of podocyte foot processes improved and were similar to those of

Figure 2

The effects of gliquidone on urinary albumin excretion rate in diabetic GK rats. (A) Effects of gliquidone on glycemic levels in diabetic GK rats. (B) Effects of gliquidone on urinary protein levels in diabetic GK rats. (C) Effects of gliquidone on urinary albumin levels in diabetic GK rats. Normal, normal rats; DN, diabetic nephropathy GK rats treated with saline; DN+Gli, diabetic nephropathy GK rats treated with gliquidone; DN+insulin, diabetic nephropathy GK rats treated with insulin. * $P < 0.05$, ** $P < 0.01$ DN+Gli vs DN group.

Figure 3

The effects of gliquidone on insulin expression in islets and C-peptide levels in serum. (A) The effect of gliquidone on insulin expression in islets. (B) The effect of gliquidone on serum C-peptide levels in diabetic rats. Normal, normal rats; DN, diabetic nephropathy GK rats treated with saline; DN+Gli, diabetic nephropathy GK rats treated with gliquidone. ** $P < 0.01$ vs DN group.

the normal group. However, after 4 weeks of insulin treatment, the morphologies of the GBM and foot processes were not obviously different from those of the diabetic nephropathy group (Fig. 4).

Effects of gliquidone on the absorption of albumin by HK-2 cells

Cultured HK-2 cells can absorb FITC-labeled human albumin from culture medium. After co-incubating with AGEs, the HK-2 cells exhibited a decreased intracellular fluorescence intensity compared with the control group. In the gliquidone group, the fluorescence intensity was stronger than in the AGEs group (Fig. 5A and B). Gliquidone increased the cellular absorption of albumin in a dose-dependent manner (Fig. 5C).

Effects of gliquidone on the expression of megalin and cubilin in kidney tubules and HK-2 cells

The expression of megalin and cubilin in the tubular tissue was decreased in the diabetic nephropathy group compared with the control group (Fig. 6A, B, and C). After 4 weeks of treatment with gliquidone, the tubular expression of megalin and cubilin was upregulated.

The expressions of megalin and cubilin were decreased in AGE-treated HK-2 cells compared with normal controls. However, gliquidone appeared to block the effects of AGE, as demonstrated by the normal expression levels of megalin and cubilin in the AGE+gliquidone group (Fig. 6D, E, and F).

Effects of gliquidone on the glomerular expression of RAGE, PKC β , and PKA

The glomerular expression of RAGE and PKC β in GK rats with diabetic nephropathy was upregulated, while the expression of PKA was markedly decreased compared with controls. The expression of RAGE and PKC β was decreased and the expression of PKA was increased in the gliquidone group compared with the diabetic nephropathy group (Fig. 7A, B, and C).

Effects of gliquidone on the expression of RAGE, PKC β , and PKA in kidney tubules and HK-2 cells

The changes in RAGE, PKC β , and PKA levels in tubular tissues of the three groups were similar to those found in the glomerulus of the three groups. The tubular expression of RAGE and PKC β in GK rats with diabetic nephropathy GK rats was increased, while PKA expression was markedly decreased, compared with normal controls, at the mRNA, protein and tissue staining levels (Fig. 8A, B, and C). In the

Figure 4

The effects of gliquidone on the structure of the GBM and on foot processes of podocytes. Normal, normal rats; DN, diabetic nephropathy GK rats treated with saline; DN+Gli, diabetic nephropathy GK rats treated with gliquidone; DN+insulin, diabetic nephropathy GK rats treated with insulin.

Figure 5

Effects of gliquidone on the absorption of albumin by HK-2 cells. (A) HK-2 cells absorbed FITC-labeled human albumin as demonstrated by microscopy. (B) The quantitative analysis of absorption as measurements of fluorescence intensity under a fluorescence plate reader and corrected

cell numbers by total protein. (C) Dose-effect of gliquidone on albumin absorption. All of the experiments were performed in triplicate. Four wells were set up for each group. ** $P < 0.01$ vs AGEs group.

gliquidone group, the tubular expression of RAGE and PKC β was lower and PKA expression was higher compared with the diabetic nephropathy group.

In HK-2 cells, the expression of RAGE and PKC β in the AGEs group was increased, while the expression of PKA was decreased compared with the normal control group. In the AGEs+gliquidone group, the expression of RAGE and PKC β was downregulated and the expression of PKA was upregulated compared with the AGEs group (Fig. 8D, E, and F).

Effects of a PKC inhibitor and PKA activator on albumin absorption function and megalin and cubilin expression in HK-2 cells

As AGEs and gliquidone affect albumin absorption, along with PKC and PKA expression, the PKC inhibitor, LY333531 and PKA activator, 8-Br-cAMP were used to investigate the effect of PKC and PKA on albumin absorption and the expression of megalin and cubilin in HK-2 cells. The results indicated that the decreased albumin absorption found in AGEs-treated HK-2 cells could be reversed by co-incubating the cells with gliquidone, LY333531 or 8-Br-cAMP, but not with insulin (Fig. 9A). The decreased expression of megalin and cubilin in AGEs-treated HK-2 cells was also reversed by

gliquidone, LY333531 and 8-Br-cAMP, but not by insulin (Fig. 9B and C).

Discussion

Diabetic nephropathy is one of the most serious complications of DM. However, the pathogenic mechanism of diabetic nephropathy has remained elusive and there is no specific, efficacious drug treatment for diabetic nephropathy. Therefore, finding an effective drug to treat diabetic nephropathy has been a shared goal of researchers in recent years.

In this study, we found that gliquidone markedly lowered protein levels in the urine and improved nephropathic lesions in GK rats with diabetic nephropathy. Improvement of diabetic nephropathy with gliquidone was closely correlated with improved glomerular filtration and tubular reabsorption of albumin. These results provide evidence supporting the use of gliquidone for the prevention and treatment of diabetic nephropathy.

DM-induced microvascular lesions are considered to be one of the major causes of diabetic nephropathy. When glucose metabolism is disturbed, the production of AGEs increases; the AGEs act on the glomerular capillary receptor (i.e., RAGE) and promote oxidative stress by

Figure 6

Effects of gliquidone on megalin and cubilin expression in kidney tubules of diabetic rats and in HK-2 cells. (A) The expression of megalin and cubilin in tubular tissue was evaluated using real-time PCR (A), western blotting (B), and immunohistochemical staining (C). The expression of

megal and cubilin in HK-2 cells was evaluated using real-time PCR (D), western blotting (E), and immunofluorescence staining (F). * $P < 0.05$, ** $P < 0.01$ vs DN group.

activating PKC, leading to an accumulation of ROS and NO that results in glomerular capillary endothelial cell injury. PKC has been reported to play a role in the pathogenesis of diabetic nephropathy, by contributing to cytokine activation, growth of extracellular matrix, cellular growth and proliferation, angiogenesis, vascular contraction and dilatation, and changes in vascular permeability (Noh & King 2007, Gerales & King 2010). Within the PKC family, the isoform PKC β has the greatest effect on the occurrence and development of diabetic nephropathy (Meier *et al.* 2009, Gerales & King 2010). The over-expression of PKC β leads to upregulation of TGF- β expression, which then induces the deposition of extracellular matrix, leading to glomerular sclerosis and tubular fibrosis (Koya *et al.* 1997, Slattery *et al.* 2008, Wu *et al.* 2009). Previous studies have demonstrated that the progression of diabetic nephropathy is delayed by knocking out the PKC β (*Prkcb*) gene or by administering a PKC β inhibitor (Tuttle *et al.* 2005, Meier *et al.* 2009). It is generally accepted that PKC activation promotes,

while PKC inhibition arrests, the occurrence of diabetic nephropathy (Noh & King 2007). Recently, we have found that PKA activity is also closely correlated with the occurrence of diabetic nephropathy. The use of a PKA agonist can control or improve diabetic nephropathy (Wang *et al.* 2012).

In this present study, we found a marked decrease in microvascular lesions, in rats with diabetic nephropathy, after treatment with gliquidone. This was manifested by a downregulation in the expression of glomerular capillary RAGE, a reduction in PKC activity and an increase in PKA activity. In addition, the thickness of the GBM decreased and the morphology and distribution of podocytes improved. Biochemical analyses demonstrated a marked decline in both the 24 h urinary protein and 24 h urinary albumin levels, indicating that gliquidone improves glomerular filtration.

Islet transplantation has been reported to prevent or reverse diabetic microvascular lesions and to delay the onset of increased urinary protein in recipients

Figure 7

Effects of gliquidone on RAGE, PKC β , and PKA expression in the glomeruli of diabetic rats. Real-time PCR (A), western blotting (B), and immunohistochemical staining (C) were used to evaluate the expression of megalin and cubilin in glomerular tissue. * $P < 0.05$, ** $P < 0.01$ vs DN group.

Figure 8

Effects of gliquidone on RAGE, PKC β , and PKA expression in kidney tubules of diabetic rats and in HK-2 cells. The expression of RAGE, PKC β , and PKA in tubular tissue was evaluated using real-time PCR (A), western blotting (B) and immunohistochemical staining (C). The expression of RAGE, PKC β ,

and PKA in HK-2 cells were evaluated using real-time PCR (D), western blotting (E), and immunofluorescence staining (F). * $P < 0.05$, ** $P < 0.01$ vs AGEs group.

(Fiorina *et al.* 2003, 2005). Recent studies have indicated that C-peptide produced by islet cells might help to prevent and treat diabetic microvascular lesions (Cotter *et al.* 2003, Forst & Kunt 2004, Hills *et al.* 2010). In fact, several human trials and animal studies have demonstrated that C-peptide can improve the kidney structural and functional abnormalities of diabetic nephropathy (Hills *et al.* 2010). Samnegard *et al.* (2005) reported a marked decrease in proteinuria and mesangial matrix synthesis in diabetic nephropathy rats treated with C-peptide compared with those treated with placebo. In this present study, gliquidone was found to elevate the level of C-peptide in the plasma (Fig. 3B) of diabetic GK rats. This may be one of the ways that gliquidone improves diabetic neuropathy.

Proteinuria is one of the major clinical manifestations of diabetic nephropathy and is closely correlated with the degree of renal lesions (Araki *et al.* 2008). The structural

alterations in glomerular filtration barriers and tubular reabsorption of protein are the major pathophysiological determinants for the occurrence of proteinuria in diabetic nephropathy. Changes in the GBM and podocytes damage the glomerular filtration barriers. The lesions in the GBM and podocytes clearly improved after gliquidone treatment and this is probably one of the reasons for the lower levels of urinary protein and albumin that were observed after treatment with gliquidone.

Reduced tubular reabsorption of proteins is another major contributor to proteinuria in diabetic nephropathy (Russo *et al.* 2009). It is known that tubular epithelial cells express RAGE on their surfaces, which conjugate with AGEs. AGEs can induce the occurrence of oxidative stress through RAGE by producing a large amount of ROS, activating the rennin-angiotensin system, interacting with signaling molecules such as PKC and nuclear factor kappa B (NF- κ B) and causing tubular interstitial fibrosis

Figure 9

Effects of a PKC inhibitor and a PKA activator on albumin absorption function and megalin and cubilin expression in HK-2 cells. (A) The effects of LY333531 (PKC inhibitor) and 8-Br-cAMP (PKA activator) on albumin absorption by HK-2 cells. * $P < 0.01$ vs control group; # $P < 0.01$ vs AGE group.

(B) The effects of LY333531 and 8-Br-cAMP on the expression of megalin and cubilin in HK-2 cells determined by real-time PCR. (C) The effects of LY333531 and 8-Br-cAMP the expression of megalin and cubilin in HK-2 cells determined by western blotting.

(Yamagishi & Matsui 2010). The proximal renal tubule is a major site of reabsorption and is particularly sensitive to DM-associated metabolites and activated factors. Therefore, changes in proximal tubular epithelial cells contribute to the occurrence of tubular lesions in diabetic nephropathy. In this study, the proximal tubular expression of RAGE was upregulated, PKC activity was increased and PKA activity was decreased in rats with diabetic nephropathy. The proximal tubular expression of RAGE was downregulated and the PKC and PKA activities were normalized in rats with diabetic nephropathy that were treated with gliquidone. Furthermore, we confirmed that AGEs were the major cause of phenotypic changes in HK-2 cells and found that gliquidone could reverse these changes.

The reabsorption of albumin in proximal tubular epithelial cells is accomplished through the effects of two receptor proteins, megalin and cubilin (Verroust *et al.* 2002). The altered expression of these proteins can affect the reabsorption of albumin (Hosojima *et al.* 2009, Cabezas *et al.* 2011). In the present study, the proximal tubular expression of megalin and cubilin were markedly downregulated in GK rats with diabetic nephropathy, however, the expression was normalized after treatment with gliquidone. These findings were further confirmed using HK-2 cells. Our results demonstrated that AGEs

lowered albumin uptake by downregulating the expression of megalin and cubilin, while gliquidone increased albumin uptake in a dose-dependent manner by upregulating the expression of megalin and cubilin. This supports the idea that gliquidone lowers the urinary protein level in diabetic nephropathy by increasing the tubular reabsorption of albumin.

It should be stressed that administration of gliquidone via a micro-osmotic pump is probably different from oral administration in terms of therapeutic efficacy. A higher plasma concentration might be obtained with the use of a micro-osmotic pump. If this is the case, the renal excretion of gliquidone will be higher and the glomerular and tubular effects might be more apparent. Gliquidone is predominantly metabolized through the portal vein system so the plasma concentration is low when it is administered orally. Indeed, after oral intake, the renal excretion concentration is estimated to be only 5% of the total concentration. As a result, it may be difficult to control diabetic nephropathy and lower urinary protein through oral administration of gliquidone.

Our results indicate that gliquidone can effectively decrease urinary protein by improving glomerular lesions and promoting tubular reabsorption in GK rats with diabetic nephropathy. Gliquidone has the potential to be an effective therapeutic drug for the treatment of diabetic

nephropathy, however pharmacokinetic differences between osmotic pump and oral administration must be taken into consideration.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0199>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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