Role of 12-lipoxygenase in decreasing P-cadherin and increasing angiotensin II type 1 receptor expression according to glomerular size in type 2 diabetic rats

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Running title: Role of 12-LO on glomerular expression of P-cadherin

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Abstract

12-lipoxygenase (12-LO) was implicated in the development of diabetic nephropathy (DN), in which the proteinuria was thought to be associated with a decreased expression of glomerular P-cadherin. Therefore we investigated the role of 12-LO in the glomerular P-cadherin expression in type 2 diabetic rats according to the glomerular sizes. Rats fed with high fat diet for 6 weeks were treated with low-dose streptozotocin. Once diabetes onset, diabetic rats were treated with 12-LO inhibitor cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC) for 8 weeks. Then glomeruli were isolated from diabetic and control rats with a sieving method. RT-PCR, Western blotting and immunofluorescent staining were used for mRNA and protein expressions of P-cadherin and angiotensin II (Ang II) type 1 receptor (AT1). We found that CDC did not affect the glucose levels, but completely attenuated diabetic increases in glomerular volume and proteinuria. Diabetes significantly decreased the P-cadherin mRNA and protein expressions and increased the AT1 mRNA and protein expressions in the glomeruli. These changes were significantly prevented by CDC and recaptured by direct infusion of 12-LO product [12(S)-HETE] to normal rats for 7 days. The decreased P-cadherin expression was similar between large and small glomeruli, but the increased AT1 expression was significantly higher in the large than the small glomeruli from diabetic and 12(S)-HETE-treated rats. Direct infusion of normal rats with Ang II for 14 days also significantly decreased the glomerular P-cadherin expression. These results suggest that diabetic proteinuria is mediated by the activation of 12-LO pathway that is partially attributed to the decreased glomerular P-cadherin expression.

Keywords: diabetic nephropathy; P-cadherin; 12-lipoxygenase; angiotensin II type 1 receptor; slit diaphragm
Introduction

Glomerular hypertrophy and proteinuria are hallmarks of the early diabetic nephropathy (DN), and can be induced by hyperglycemia and several growth factors such as angiotensin II (Ang II) via their specific receptors (19). There are two Ang II plasma receptors, referred to as type 1 receptor (AT1) and type 2 receptor subtypes (6). Most of the known effects of Ang II in the adult tissues are attributable to AT1 intracellular signaling (6, 20, 25). The expression of AT1 is thus important for the biological effect of Ang II. A number of clinical and experimental studies have demonstrated that AT1 antagonist reduce the damage of renal tissue and decrease proteinuria (3, 5, 26). One of the renal hemodynamic effects of Ang II was assumed as the increase in the products of the 12-lipoxygenase (12-LO) pathway (2). To support this early study (2), we recently demonstrated that the up-regulation of AT1 is associated with the activated 12-LO pathway in the diabetic kidneys and the mesangial cells exposed to high levels of glucose (27, 29).

The 12-LO pathway derives several products to exert numerous physiological and pathological effects in the kidney. One of 12-LO products is the formation of oxidized lipids such as 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE]. Both 12-LO and 12(S)-HETE were implicated in DN (10, 22, 27, 29). However, how 12-LO induces renal dysfunction remains under investigation.

The pathological change responsible for proteinuria in various glomerular diseases, including DN, predominantly is due to the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier. The urinary side of the capillary loop in the glomerulus is covered by highly branched glomerular visceral epithelial cells, called podocytes (21). Evidence indicates that the podocyte slit diaphragm to provide a structural framework for the filtration barrier is also essential as a signaling platform (12). In the past, the slit diaphragm was considered as a modified
tight junction due to the presence of the tight junction protein, zonula occludens-1, at the cytoplasmic side of the slit diaphragm (14). However, recent studies using immunofluorescent and immunoelectron microscope revealed that the slit diaphragm is a modified adherens junction, rather than a modified tight junction, and that, in addition to nephrin, NEPH1, and FAT, the P-cadherin was also localized at the slit diaphragm (7, 23).

The contribution of changes in nephrin expression to proteinuria in DN has been investigated. Bonnet et al. found a reduction in nephrin mRNA and protein expressions in streptozotocin (STZ)-induced diabetic spontaneously hypertensive rats at 32 weeks after diabetes onset (4). Aaltonen et al. observed an increase in nephrin mRNA levels in STZ-induced diabetic rats and in non-obese diabetic mice before the development of significant albuminuria (1). Studies showed that the prevention of the decreased nephrin expression with AT1 antagonist can significantly ameliorate proteinuria levels (24). All these studies suggest the important role of slit diaphragm in the maintenance of the normal glomerular filtration. Furthermore, Kim et al. demonstrated that nephrin mRNA and protein expression were decreased in hypertrophied glomeruli whereas nephrin expression is increased in the relatively smaller glomeruli at the early stage of diabetes (13). This study suggests that the decreased expression of nephrin in the hypertrophied glomeruli may be responsible for the albuminuria at the early stage of diabetes.

Besides nephrin, P-cadherin as one of the key components of the slit diaphragm was also found to play critical role in the maintenance of the normal glomerular filtration. In non-diabetic condition, proteinuria was found related to the depression of P-cadherin expression (16). It is also reported that the detrimental effect of adriamycin on podocyte permeability is associated with the disruption of the tight junctions by decreasing P-cadherin expression (15). We have demonstrated that the decrease in P-cadherin expression in the diabetic kidney and in the podocytes exposed to
high levels of glucose in vitro (28). However, whether the decrease of P-cadherin expression varies according to the sizes of glomeruli under diabetic conditions and whether the decreased expression of P-cadherin in different sizes of glomeruli is related to diabetic activation of 12-LO pathway needs to be investigated.

The present study was thus to test a hypothesis that 12-LO inhibition may ameliorate proteinuria by preventing the reduction of glomerular P-cadherin expression caused by diabetes. To this end, we have used a type 2 diabetic rat model that was induced by high-fat diet (HFD) for 6 weeks, followed by a treatment with low-dose STZ. Eight weeks after hyperglycemia glomeruli were isolated from both diabetic and control rats and separated into hypertrophied (large size, LG) and relative small size (SG) groups. The P-cadherin expression according to the sizes of isolated glomeruli was examined at mRNA and protein levels. To define the role of 12-LO in diabetic proteinuria and P-cadherin expression, 12-LO inhibitor cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC) was used to treat the type 2 diabetic rats for 8 weeks. In addition, to define the direct roles of 12-LO metabolic pathway and Ang II in the decrease of glomerular P-cadherin expression, 12-LO product 12(S)-HETE and Ang II were infused into normal rats, respectively.

MATERIALS AND METHODS

Materials. The 12(S)-HETE and cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) were purchased from Biomol (Plymouth Meeting, PA, USA). We purchased the STZ, Ang II and β-actin antibody from Sigma (St Louis, MO, USA), P-cadherin and AT1 antibody and goat anti-rabbit IgG-HRP from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and supersignal chemiluminescent reagent from Pierce (Rockford, IL, USA). The relative multiplex reverse transcription-polymerase chain reaction (RT-PCR) kits from Ambion Inc (Austin, TX, USA), the
RNA STAT-60 reagents from Tel-Test (Friendswood, TX, USA), the 12(S)-HETE EIA Kit from Assay Designs (Ann Arbor, MI, USA), and the urinary albumin ELISA Kit from Exocell Inc (Philadelphia, PA, USA) were bought. We purchased the osmotic mini-pump (Alzet Model 1002) from DURECT Corp (Cupertino, CA, USA), the TINA image software (Raytest, Straubenhardt, Germany), digital image analysis (MetaMorph version 4.6r5) from Universal Imaging Corp. (Downingtown, PA, USA), and the PRISM software from Graph Pad (San Diego, CA, USA).

Animal models. All animal studies were conducted following the protocol approved by the committee for the care and use of laboratory animals of Jilin University. The animals were housed in a temperature-controlled room and given free access to the water. Male Wistar rats with 200-220g body weight were used for the following three animal models:

(1) Type 2 diabetes model. Rats were randomly assigned to either regular rat chow (Ctrl, N=10) or 60% HFD (N=24) for 6 weeks based on published studies (8, 18). After 6-week HFD, rats were received with either a low-dose STZ (35 mg/kg) to induce hyperglycemia or citrate buffer (as control). STZ was dissolved in citrate buffer (0.01 mol/l, pH 4.5) and injected intraperitoneally. Blood glucose levels were measured 3 days after the STZ injection to confirm the development of diabetes. Hyperglycemic rats were given with and without CDC treatment at 4 mg/kg subcutaneously in the hind leg every other day for 8 weeks (N=10). CDC was dissolved in sesame oil and its dose used in this study was selected based on our previous studies (29). For the CDC study, diabetic rats were injected with CDC mix in sesame oil as diabetes/CDC group (CDC) or only with diluent sesame oil as diabetic group (DN). At sacrificing time, glomeruli were isolated from part of the kidney and stored at –70°C for further study. The rest kidney specimens were fixed in 10% formalin for histological evaluation or snap-frozen in liquid nitrogen for biochemical studies. Blood glucose was measured by glucometer and 24-h urinary albumin
excretion was determined by ELISA kit.

(2) 12(S)-HETE infusion model. Sixteen male Wistar rats were randomly assigned to receive either 12(S)-HETE infusion at a rate of 1 mg/kg daily or vehicle (normal saline) for 7 days by osmotic mini-pumps (N=8). For implanting the osmotic mini-pumps the rats were anesthetized with isoflurane and an incision was made on rat back to subcutaneously insert the osmotic mini-pumps under sterile condition. After 7-day infusion of 12(S)-HETE the rats were sacrificed for isolation of glomeruli as described in the above study.

(3) Ang II infusion model. Sixteen male Wistar rats were randomly assigned to receive either Ang II infusion at a rate of 400 ng/kg min or vehicle (ethanolamine) by osmotic mini-pumps for 14 days (N=8). Osmotic mini-pumps were implanted as described above and the glomerular isolation was same as the above two studies.

Glomerular isolation. Glomeruli were isolated with a sieving method using sieves with pore sizes of 250, 150, 125, and 75 \( \mu m \), based on our previous study (13). The glomeruli were collected under an inverted microscope to minimize tubular contamination. We classified glomeruli into small glomeruli (on the 75 \( \mu m \) sieve, SG) and large glomeruli (on the 125 \( \mu m \) sieve, LG) groups. Then glomeruli were frozen in liquid nitrogen and stored at –70 °C.

Morphometric measurement of glomerular volume. Glomerular volume (VG) was calculated as previously described (10). Briefly, photographs of 50 glomeruli were taken using a digital camera at the time of sieving and the surface areas were traced using a computer-assisted color image analyzer. VG was calculated using the equation: \( VG=\frac{4}{3}\pi \left(\frac{Area}{\pi}\right)^{3/2} \).

Measurement of 12(S)-HETE. Glomeruli were lysed in sodium dodecyl sulfate sample buffer and the supernatants from glomerular lysate were stored at –70 °C. The levels of 12(S)-HETE in the SGs and LGs were measured using a commercial EIA kit according to the
manufacturer’s instructions and protein content was measured with the modified Lowry method.

**Relative and competitive RT-PCR.** Total RNA was isolated from isolated glomeruli using RNA-STAT 60 reagents. The preparation of cDNA, relative multiplex PCR and quantitative competitive PCRs were performed as described previously (27). Briefly, cDNA was synthesized with 1 μg of RNA using MuLV reverse transcriptase and random hexamers. Relative multiplex PCRs were performed using gene specific primers along with primers for the GAPDH RNA as an internal control. The primers used are summarized in Table 1. In some experiments we used quantitative competitive RT-PCR to determine AT1 mRNA expression using the AT1 competitor cDNA (212 bp) as internal standard. The AT1 competitor cDNA used as internal standard was designed to contain the same base pair sequence as the target cDNA that would allow efficient priming, but had a portion deleted so that the competitor PCR-generated fragment could be easily distinguished electrophoretically by size (Fig 1). The RT-PCR products were separated by electrophoresis and competitive PCR measurements were expressed with a ratio of the wild-type divided by the competitor band densities.

**Western blot analysis.** Glomeruli on 75 μm and 125 μm sieves were lysed in sodium dodecyl sulfate sample buffer. Lysate was centrifuged at 12000 rpm for 30 min at 4 °C and the supernatants were stored at -70 °C. Fifty μg proteins were loaded per lane, separated by 8% SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted with antibody to AT1 (1:1000), P-cadherin (1:1000) or β-actin (1:3000). After washing, membranes were incubated for 1 h with horseradish peroxidase-linked secondary antibody (1:2000) and immunoreactive proteins detected by Supersignal chemiluminescent reagent. Immunoblots were scanned using densitometer and the protein bands were quantified with TINA image software.

**Kidney histology.** Renal cortical slices were snap-frozen in OCT compound and sectioned
immediately for immunofluorescent staining. Briefly, for P-cadherin staining, monoclonal anti-P-cadherin antibody was diluted 1:50 with 2 % casein in bovine serum albumin and was applied for 1 h incubation at 4 °C. After washing, a secondary goat anti-rabbit IgG-FITC was added for 1 h.

Statistic analysis. Data are expressed as mean ± SD of multiple experiments. Paired Student’s t-tests were used to compare two groups or ANOVA for multiple groups, followed by Dunnet’s post test using PRISM software. Statistical significance was detected at the 0.05 level.

RESULTS

Effect of 12-LO inhibitor on the renal function of type 2 diabetic rats. Rats with six-week HFD were given one injection of low-dose STZ, which induced hyperglycemia on day 3 (fasting blood glucose levels >300 mg/dl). Diabetic rats had elevated blood glucose levels throughout the study period compared to control rats (Fig. 2A). Rats with 6-week HFD feeding have significantly higher body weight before STZ treatment than those with normal diet. After STZ treatment, diabetic rats did not significantly gain the body weight while the rats with normal diet continually gained the body weight so that the body weights in control and diabetic groups were not significantly different at the termination of experiment (Fig. 2B). The untreated diabetic rats had significant increases in the kidney weight/body weight ratio (Fig. 2C), the glomerular volume (Fig. 2D), and the proteinuria (Fig. 2E) compared with the control rats.

CDC treatment significantly prevented diabetic increases in the kidney weight/body weight ratio, the glomerular volume and the proteinuria without influence of the body weight or blood glucose levels (Fig. 2).
Effect of 12-LO inhibitor on the glomerular P-cadherin expression of type 2 diabetic rats. A significant decrease in glomerular P-cadherin mRNA expression was observed in diabetic rats compared with controls (p<0.01) without significant difference between LG and SG groups (Fig. 3A). Similarly, Western blotting also showed a significant decrease in the P-cadherin protein expression without significant difference between two size groups of glomeruli (Fig. 3B). Immunofluorescent staining for whole kidney confirmed the glomerular specific expression of P-cadherin and the decreased expression of P-cadherin in diabetic glomeruli (Fig. 3C). CDC treatment partially, but significantly prevented diabetic decrease in the P-cadherin expression.

Effect of 12-LO inhibitor in the glomerular AT1 expression of type 2 diabetic rats. A significant increase in the glomerular AT1 mRNA expression, detected by RT-PCR (Fig. 4A), was observed in diabetic rats compared with the controls (p<0.01). The AT1 expression was significantly higher in the LG group than that in the SG group (Fig 4A, p<0.05). To ensure the accurately reflect the difference for AT1 mRNA expression between the LGs and the SGs, the competitive RT-PCR with AT1 specific primers was performed. As an internal standard, an AT1 deletion mutant (competitor) that is co-amplified with the endogenous gene was used. Using the same primers for the mutant and for the endogenous gene ensures comparable amplification efficiencies. Each reaction was performed with a fixed amount of AT1 wild type cDNA and a fixed amount of AT1 competitor cDNA. Data are expressed as the ratio of wild type to competitor optical densities measured by densitometor. Competitive RT-PCR data also showed a significant increase in AT1 mRNA expression in the LG group compared to the SG group (Fig 4B, p<0.05). In consistence with mRNA finding, Western blotting showed the significant increase in AT1 protein expression with a higher expression in the LGs than that in the SGs of diabetic rats (Fig 4C). CDC treatment significantly prevented diabetic increase in the expression of AT1 at both
mRNA and protein levels (Fig. 4).

12(S)-HETE levels in type 2 diabetic glomeruli. We have previously shown that 12-LO product 12(S)-HETE levels were significantly increased in whole kidney under diabetic conditions (27). Here we thus have tested 12(S)-HETE levels in the SGs and LGs under diabetic conditions by EIA. As expected, the levels of 12(S)-HETE in glomeruli were significantly increased in diabetic rats compared to controls. The level of 12(S)-HETE is significantly higher in the LGs than that in the SGs (Fig. 5, \( p<0.05 \)). CDC could significantly attenuated diabetic increase in the levels of 12(S)-HETE.

Effect of 12(S)-HETE infusion on the glomerular expression of P-cadherin and AT1. To define the causative role of 12(S)-HETE in diabetic decrease in the glomerular P-cadherin expression, normal rats were infused with 12(S)-HETE for 7 days. Direct infusion of 12(S)-HETE caused a significant decrease in glomerular P-cadherin mRNA (Fig. 6A) and protein (Fig. 6B) expression without a significant difference between LG and SG groups.

Furthermore, we examined whether infusion of 12(S)-HETE also induces the glomerular AT1 expression as observed in diabetic glomeruli (Fig. 4). Similarly, subcutaneous infusion of 12(S)-HETE caused a significant increase in the glomerular AT1 mRNA (Fig. 7A) and protein (Fig. 7B) expression. The expression of AT1 at both mRNA and protein levels was higher in the LG group than that in the SG group (Fig. 7).

Effect of Ang II infusion on the glomerular P-cadherin expression. Next we examined the change of glomerular P-cadherin mRNA and protein expression in the normal rats that were directly infused with Ang II for 14 days. Subcutaneous infusion of Ang II significantly decreased the glomerular expression of P-cadherin mRNA (Fig. 8A) and protein (Fig. 8B) without significant difference between the LGs and the SGs.
DISCUSSION

P-cadherin is one of the classical cadherins, a superfamily of glycoproteins involved in cell-cell adhesion (9, 23). Based on the location of P-cadherin at the slit diaphragm, it has been proposed to function as a basic scaffold for the slit diaphragm, whereas the permselectivity is provided by the slit diaphragm complex composed of P-cadherin and other proteins, such as nephrin (23). Previous studies have indicated the potential role of decreased P-cadherin in the development of proteinuria in non-diabetic conditions (15, 16). Furthermore, reportedly intravenous injection of anti-P-cadherin antibody resulted in a 49% increase in urinary protein excretion independent of nephrin or NEPH1 (17), confirming the important role of P-cadherin in maintaining the glomerular filtration barrier. We have previously demonstrated the decreased expression of P-cadherin in the diabetic kidneys and the podocytes exposed to high levels of glucose in vitro (28), which was further supported by the present study to show the decreased expression of P-cadherin mRNA and protein specific in the glomeruli (Fig. 3C). Furthermore, we also provide the following new findings.

First we demonstrated that diabetic decrease in the glomerular expression of P-cadherin is partially mediated by the activation of 12-LO pathways. In a type 2 diabetic rat model, we found that inhibition of 12-LO pathways with its inhibitor can partially, but significantly, attenuate diabetic decrease in the glomerular expression of P-cadherin (Fig. 3) along with an almost complete improvement of renal dysfunction (Fig. 2). Direct infusion of 12-LO product 12(S)-HETE to normal rats also significantly decreased the glomerular expression of P-cadherin at both mRNA and protein levels (Fig. 6). These results suggest that activation of 12-LO pathway plays critical roles in the development of DN by multiple pathogenic mechanisms (11, 22), one of which is the decreased expression of glomerular P-cadherin.
The second new finding is that diabetic decrease in the glomerular expression of P-cadherin is independent of glomerular sizes. We demonstrated for the first time that no statistical difference between the LGs and the SGs was found for the decreased P-cadherin expression no matter induced by diabetes (Fig. 3), 12(S)-HETE (Fig. 6) or Ang II (Fig. 8). Several studies demonstrated that diabetic glomerular sizes were different by examination with microdissection or a sieving technique. However, most of the previous studies that have focused on changes in glomerular nephrin showed inconsistent results. Bonnet et al. found a reduction of the glomerular expression of nephrin mRNA and protein in STZ-induced diabetic and spontaneously hypertensive rats (4), whereas another study in STZ-induced diabetic rats and in non-obese diabetic mice revealed an increase in nephrin mRNA levels (1). Kim et al. found an increased nephrin expression in the SGs and a decreased nephrin expression in the LGs under diabetic group compared with controls (13). Based on the finding that nephrin expression is diminished in the LGs, they assumed that albuminuria may arise first in the hypertrophied glomeruli, i.e.: LGs, at the early stage of DN (13). In a line with this finding we found that P-cadherin expression are significantly decreased in type 2 diabetic glomeruli, but we did not found the significant difference for the decreased P-cadherin expression between the LGs and the SGs under type 2 diabetic conditions. This suggests that the profiles of P-cadherin and nephrin expressions in diabetic glomeruli may be different: Nephrin expression varies, but P-cadherin expression does not vary, among the different sizes of glomeruli under diabetic conditions. Therefore, we proposed that urinary protein excretion is partially dependent of the decreased expression of nephrin that changes according to the sizes of glomeruli, and also partially dependent of the decreased P-cadherin expression that does not change according to sizes of glomeruli. This will explain why in the previous studies the changes of glomerular nephrin expression did not
consistently parallel proteinuria results (1, 4, 13).

The third new finding of the present study is the direct role of 12-LO in diabetic up-regulation of AT1 expression in both LGs and SGs. As shown in Fig. 4, the levels of AT1 mRNA and protein levels are significantly higher in the LGs than that in the SGs, which could be almost completely prevented by CDC. Interestingly 12(S)-HETE level was also higher in the LGs than that in the SGs (Fig. 5). Direct infusion of 12(S)-HETE to normal rats increased the glomerular AT1 expression, which is also significantly higher in the LGs than the SGs (Fig. 7). These direct correlation findings suggest that 12(S)-HETE may be the direct causative of glomerular AT1 up-regulation.

Finally, we demonstrated that 12(S)-HETE-mediated diabetic suppression of glomerular P-cadherin expression is accompanied with up-regulation of AT1 expression. We found that diabetic renal dysfunction paralleled the up-regulated AT1 expression, particularly in the LGs. Inhibition of 12-LO pathways almost completely prevented both renal dysfunction and up-regulated glomerular AT1 expression. The fact that like diabetes, direct infusion of 12(S)-HETE to normal rats induced a significant decrease in glomerular P-cadherin expression (Fig. 6) along with a significant increase in AT1 up-regulation (Fig. 7) suggests the possible involvement of Ang II in the diabetic suppression of glomerular P-cadherin. This was confirmed by the finding that glomerular P-cadherin expression was significantly decreased in rats with Ang II infusion (Fig. 8). However, the pattern of increased AT1 expression in different sizes of glomeruli was not directly correlated with the decreased expression of P-cadherin according the different sizes of glomeruli. For instance, diabetes- and 12(S)-HETE-increased AT1 expression is higher in the LG group than the SG group (Fig. 4 and Fig. 7), but the P-cadherin expression in the glomeruli was decreased without significant difference according to the sizes of glomeruli in
diabetic (Fig. 3), 12(S)-HETE-treated (Fig. 6) and even Ang II-treated rats (Fig. 8). Therefore, diabetes-induced P-cadherin down-regulation may be partially associated with Ang II/AT1 signaling pathway.

In summary, the present study has investigated the effect of 12-LO on the glomeruli P-cadherin expression with a focus on the profile in different sizes of glomeruli under type 2 diabetic conditions. Using HFD/STZ-induced diabetic rat model, we demonstrated for the first time that the glomerular expression of P-cadherin at both mRNA and protein levels was significantly decreased in diabetic groups, which was significantly prevented by inhibition of 12-LO. Like diabetes direct infusion of 12-LO product to normal rats for 7 days also significantly decreased the glomerular expression of P-cadherin. Direct infusion of Ang II can also decrease glomerular P-cadherin expression. However, there was no significant difference for the P-cadherin expression between different sizes of glomeruli under diabetic condition. All these results suggest that diabetic proteinuria is mediated by the activation of 12-LO pathway. The decreased glomerular P-cadherin expression is probably partial contribution to the 12-LO pathogenic effect on DN.

Grants

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16. Li Y, Kang YS, Dai C, Kiss LP, Wen X, Liu Y. Epithelial-to-mesenchymal transition is a


LEGENDS

Fig. 1. The nucleotide sequence of rat AT1 competitor. As an internal standard, an AT1 deletion mutant (competitor) that is co-amplified with the endogenous gene was used in this study. Using the same primers for the mutant and the endogenous genes ensures comparable amplification efficiencies. The nucleotides used as primers for RT-PCR are indicated by grey box.

Fig. 2. Clinical characteristics of rats at the end of the study. Blood glucose levels (A), body weight (B), kidney weight/body weight ratio (C), glomerular volume (D), and urinary albumin excretion (E) were measured at end of the study. The data shown are the mean ± SD (N=10 in each group). a, p<0.01 vs. Ctrl; b, p<0.05 vs. Ctrl; c, p<0.05 vs. DN; d, p<0.01 vs. DN.

Fig. 3. Glomerular P-cadherin expression. P-cadherin mRNA (A) and protein (B) expressions in the SGs and LGs were detected by semi-quantitative RT-PCR and Western blot, respectively. Immunofluorescent staining for the P-cadherin (C) was performed with the kidney tissue. A significant decrease in glomerular P-cadherin expression was exhibited with linear/punctuate distribution along the glomerular capillary loops in DN rats (C, × 400). The data shown are the mean ± SD (N=10 in each group). a, p<0.01 vs. corresponding Ctrl; b, p<0.05 vs. corresponding DNs.

Fig. 4. Glomerular AT1 expression. AT1 mRNA expressions in the SGs and LGs were detected by semi-quantitative RT-PCR (A) and quantitative competitive RT-PCR (B); AT1 protein in the SGs and LGs were determined by Western blot (C). The data shown are the mean ± SD (N=10 in each group). a, p<0.05 vs. corresponding Ctrl; b, p<0.05 vs. corresponding DN-SGs; c, p<0.05 vs. corresponding DNs.
Fig. 5. The levels of glomerular 12(S)-HETE. The levels of glomerular 12(S)-HETE were detected by EIA as described in the Materials and Methods. The data shown are the mean ± SD (N=10 in each group). a, $p<0.01$ vs. correspondingCtrls; b, $p<0.05$ vs. corresponding DN-SGs; c, $p<0.05$ vs. corresponding DNs.

Fig. 6. Effects of 12(S)-HETE infusion on glomerular P-cadherin expression. P-cadherin mRNA expressions (A) and protein (B) in the SGs and LGs were detected by semi-quantitative RT-PCR and Western blotting, respectively. The data shown are the mean ± SD (N=8 in each group). a, $p<0.01$ vs. correspondingCtrls.

Fig. 7. Effects of 12(S)-HETE infusion on the glomerular AT1 expression. AT1 mRNA (A) and protein (B) expression in the SGs and LGs were detected by semi-quantitative RT-PCR and Western blot, respectively. The data shown are the mean ± SD (N=8 in each group). a, $p<0.05$ vs. correspondingCtrls; b, $p<0.05$ vs. HETE-SG..

Fig. 8. Effects of Ang II infusion on the glomerular P-cadherin expression. P-cadherin mRNA (A) and protein (B) expressions in the SGs and LGs were detected by semi-quantitative RT-PCR and Western blot, respectively. The data shown are the mean ± SD (N=8 in each group). a, $p<0.01$ vs. correspondingCtrls.
### Table 1 The sequences of the PCR primer used in this study

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GGCCATCGTCCACCCAATGAAGTCTCG
CCTTCGCCGCACGATGCTGGTGCGGCAAA
AGTCACCTGCATCATCATCTCTGGCTGAT
GGCTGGCGTTGGCCAGTGGTGCCAGC-3'

Fig. 1
Fig. 2

A. Blood glucose (mg/dl)

B. Body weight (g)

C. Kidney/body weight (mg/g)

D. Glomerular volume (x10^6 μm³)

E. Urinary albumin excretion (mg/d)

Legend:
- Ctrl
- DN
- CDC

Significance levels:
- a
- b
- c
- d
Fig. 3

A

Ctrl DN CDC

P-cadherin(194bp)

GAPDH(538bp)

B

Ctrl DN CDC

P-cadherin

β-actin

C

Ctrl DN CDC
Fig. 5
Fig. 6

A

B

P-cadherin/GAPDH

P-cadherin/Actin
Fig. 7

A

AT1/GAPDH

AT1

GAPDH

0.0 0.6 1.2 1.8 2.4 3.0

Ctrl-SG Ctrl-LG HETE-SG HETE-LG

AT1(317bp)

GAPDH(538bp)

B

AT1

β-actin

AT1/Actin

0.0 0.6 1.2 1.8 2.4 3.0

Ctrl-SG Ctrl-LG HETE-SG HETE-LG

a, b

a

Ctrl

HETE

a, b

a

Ctrl

HETE

a, b

a

Ctrl

HETE