Original article

Acortatarin A inhibits high glucose-induced extracellular matrix production in mesangial cells

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Keywords: *acortatarin A; high glucose; mesangial cells; extracellular matrix*

Background Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Various treatment regimens and combinations of therapies provide only partial renoprotection. Therefore new approaches are needed to retard the progression of DN. The aim of the present study was to evaluate the role of a novel spiroalkaloid from *Acorus tatarinowii* named acortatarin A (AcorA) in inhibiting high glucose-induced extracellular matrix accumulation in mesangial cells (MCs).

Methods The cytotoxity of AcorA on MCs was examined by 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. The expression of fibronectin and collagen IV was examined by real time PCR and western blotting. The expression of $p22^{phox}$ and $p47^{phox}$ was detected by western blot. The interaction between $p22^{phox}$ and $p47^{phox}$ was examined by co-immunoprecipitation. The phosphorylation of $p47^{phox}$ was examined by immunoprecipitation. The phosphorylation of protein kinase C (PKC) α, PKCβ, phospholiase C gamma (PLCγ1), and the p85 subunit of PI3K was determined by Western blotting.

Results AcorA significantly inhibited high glucose-induced activation of NADPH oxidase, a ROS-generating enzyme, by increasing phosphorylation of $p47^{phox}$ and enhancing interaction between $p22^{phox}$ and $p47^{phox}$. Preincubation of AcorA with MCs inhibited high glucose-induced collagen IV and fibronectin production in a dose-dependent manner. Moreover, AcorA attenuated high glucose enhanced phosphorylation of PKCα, PKCβ, PLCγ1, and the p85 subunit of PI3K.

Conclusion AcorA inhibits high glucose-induced extracellular matrix production via blocking NADPH oxidase activation.

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The prevalence of diabetes mellitus has been increasing worldwide in the last decade. Diabetic nephropathy (DN) is a serious complication of diabetes mellitus and is the most common cause of end-stage renal disease.[1] Several interventions, such as tight glycemic control and antihypertensive therapy, especially angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers, have been shown to slow the progression of diabetes.[2-4] Nevertheless, DN remains a major long-term complication of both type 1 and 2 diabetes, because treatment commenced after the manifestation of overt clinical nephropathy often does not arrest progression to end stage renal disease. Therefore, identifying new agents to arrest further progression of the disease remains an unmet daunting task.

Excessive accumulation of extracellular matrix (ECM) in the glomerular mesangium is the major pathologic feature in DN and contributes to glomerulosclerosis.[5-7] When exposed to high glucose, mesangial cells (MCs) synthesize ECM proteins including collagens IV and fibronectin. Mesangial deposition of ECM closely correlates with deterioration of renal function[8,9] and therefore has been considered as a crucial therapeutic target of DN.[10,11] Many studies have demonstrated that ROS generation is an early response of MCs to high glucose and contributes to overproduction of ECM.[12-14]

Acorus tatarinowii is a perennial plant (Araceae) spreading over southern area of China, India, and Thailand. The rhizome of *Acorus tatarinowii* is one kind of traditional Chinese herb with aroma, pungent, and bitter sapor. Its distinct effects on calmness, anticonvulsion, intelligence improvement, and rheumatism have received great attention.[15-18] Recently, we isolated a structurally novel alkaloid named AcorA from *Acorus tatarinowii* and found that AcorA could inhibit high glucose induced ROS generation.[19] The present study was to evaluate the protecting effects of AcorA on hyperglycemia induced ECM accumulation in

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cultured MCs.

METHODS

Cell culture

Rat glomerular MCs (HBZY-1, purchased from Life-Science Academy of Wuhan University, Wuhan, China) were cultured and maintained in DMEM (Invitrogen, Carlsbad, USA), PH7.4, supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37°C.[20]

To examine the effect of AcorA, HBZY-1 cells were pre-treated with indicated concentration of AcorA at 37°C for 1 hour, and then exposed to either 5.6 (normal glucose, NG) or 30 mmol/L (high glucose, HG) D-glucose for 24 hours.[21]

MTT assay

HBZY-1 Cells were seeded into 96-well plates in a volume of 200 μl per well (1×10^5 cells/ml) and incubated for 24 hours to allow cells to attach. The cells were then incubated with indicated amount of AcorA for 1 hour. Cell viability was determined by addition of 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) at a concentration of 5 mg/ml. After incubation for 4 hours, the medium was removed and 150 μl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 540 nm by using iMark™ Microplate Reader (Bio-Rad).

Real-time PCR

Total RNA was prepared by using a TRIzol RNA isolation system according to the manufacturer's instruction. The first strand of cDNA was synthesized using 1 μg of RNA in 20 μl of reaction buffer, MMLV-RT, and random primers at 37°C for 50 minutes. Real-time PCR was performed by using a Plantinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The primer sequences are as follows: rat fibronectin, 5′-GTGGCTGCCTTCAACTTCTC-3′ and 5′-GTGGGTTGCAAACCTTCAAT-3′; rat collagen 4A1: 5′-ATTCCTTTGTGATGCACACCAG-3′ and 5′-AAGCTGTAAGCATTCGCGTAGTA-3′; rat GAPDH, 5′-ATGGCACAGTCAAGGCTGAGA-3′ and 5′-CGCTCCTGGAAGATGGTGAT-3′.

Western blotting analysis

Western blotting was performed as described previously.[22] Briefly, whole cell proteins were extracted using lysis buffer (Cell Signaling, MA, USA) and quantified by the Bradford assay (Bio-Rad, Hercules, CA, USA). Equivalent amount of proteins were resolved with SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and then incubated with rabbit anti-Fibronectin pAb (Santa Cruz Biotechnology, CA, USA), anti-Collagen IV (Abcam, MA, USA), rabbit anti-$p47^{phox}$, rabbit anti-$p22^{phox}$ (all from Santa Cruz Biotechnology), anti-p-PKCα (phospho-T638) mAb (Abcam), anti-PKCα pAb (Cell Signaling Technology), anti-p-PKCβ1 pAb (Thr-642) (Santa Cruz Biotechnology), anti-PKCβ1 pAb (Santa Cruz Biotechnology), rabbit anti-p-PLCγ1(Tyr783) pAb and anti-PLCγ1 pAb, rabbit anti-p-PI3K p85 (Tyr458) pAb, anti-PI3K p85 pAb, and rabbit anti-β-actin pAb (all from Cell Signaling) at 4°C overnight. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibodies and detected by using ECL detection system. The density of the bands was quantified by a densitometry (University Hood 2; Bio-Rad, Milan, Italy).

Co-immunoprecipitation

The binding activity of $p47^{phox}$ with $p22^{phox}$, and phosphorylation of $p47^{phox}$ in cultured MCs were determined by co-immunoprecipitation as previously described.[23] Briefly, the immunocomplexes were obtained by incubating cell lysates with rabbit anti-mouse $p22^{phox}$ and polyclonal rabbit anti-$p47^{phox}$ antibody (all from Santa Cruz), separately. Immunoblotting was performed using anti-rat $p47^{phox}$ antibody (Santa Cruz Biotechnology) and theHRP-conjugated rabbit anti-phosphoserine antibody (Stressgen Bioreagents Corp. Victoria, BC, Canada) as the primary antibody, and the HRP-conjugated swine anti-rabbit IgG (Dako Cytomation, Denmark) as the secondary antibody. To determine the total $p22^{phox}$ and $p47^{phox}$, membranes were eluted and incubated with the anti-rat $p22^{phox}$ and $p47^{phox}$ antibodies and then detected with the HRP-conjugated anti-rabbit IgG (Dako Cytomation).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) with SPSS17.0 (SPSS Inc., USA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Evaluation of the cytotoxicity of AcorA

We first examined the cytotoxicity of AcorA in cultured MCs by MTT assay. HBZY-1 cells were incubated with indicated amount of AcorA for 1 hour before analysis. As shown in Figure 1, compared with vehicle control, no obvious cell mortality was observed when cells were incubated with up to 50 μmol/L of AcorA. Therefore, we used 0 to 50 μmol/L of AcorA for the following experiments.

AcorA inhibited high glucose-induced NADPH oxidase activation

We previously reported that AcorA could inhibit high glucose-induced ROS production. Since NADPH oxidase is the predominant enzyme source for ROS generation,[24,25] we examined the effect of AcorA on the activation of ROS-generating enzyme NADPH oxidase. In line with previous reports,[26,27] 30 mmol/L glucose (high glucose) treatment resulted in a significant increase

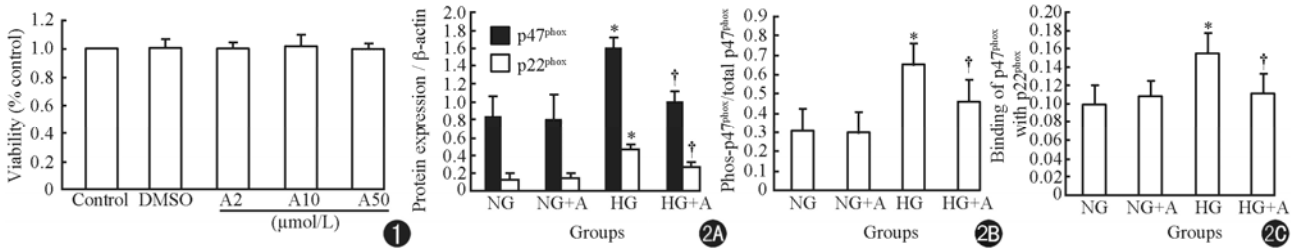

Figure 1. Cytotoxity of AcorA by MTT assay. Mesangial cells were incubated with 2, 10 or 50 μmol/L of AcorA for 2 hours and then washed for MTT assay. Data are expressed as mean±SD of three independent experiments. Control: untreated normal cells; DMSO: cells treated with vehicle; A: cells treated with AcorA.

Figure 2. AcorA attenuating effect on high glucose induced NADPH oxidase activation. **2A:** the protein level of $p47^{phox}$ and $p22^{phox}$ analyzed by Western blotting. **2B:** Phosphorylation of $p47^{phox}$ assayed by immunoprecipitation using anti-$p47^{phox}$ antibody followed by immunoblotting with antibody against phosphoserine. **2C:** The interaction of $p47^{phox}$ with $p22^{phox}$ determined by immunoprecipitation using anti-$p22^{phox}$ antibody followed by immunoblotting with anti-$p47^{phox}$ antibody. HBZY-1 cells were pre-incubated with or without 10 μmol/L AcorA for 1 hour before 30 mmol/L of glucose was added. At 12 hours after incubation, cells were harvested. $^{*}P$ <0.05 versus control cells cultured in normal medium. $^{\dagger}P$ <0.05 versus 30 mmol/L glucose treated cells. Data are expressed as mean±SD of three independent experiments. NG: cells treated with 5.6 mmol/L glucose; HG: cells treated with 30 mmol/L glucose; NG+A: cells treated with 5.6 mmol/L glucose plus AcorA; HG+A: cells treated with 30 mmol/L glucose plus AcorA.

of $p22^{phox}$ and $p47^{phox}$ expression, which was ameliorated by pre-incubation with 10 μmol/L AcorA (Figure 2A). Similarliy, high glucose induced phosphorylation of $p47^{phox}$, which was ameliorated by pre-incubation with AcorA (Figure 2B). We next examined the interaction between activated $p47^{phox}$ and membrane subunits $p22^{phox}$ by co-immunoprecipitation. As shown in Figure 2C, the binding of $p47^{phox}$ to $p22^{phox}$ was markedly increased in high glucose-stimulated HBZY-1 cells, which whereas was attenuated by pre-incubation with AcorA. Altogether, these data indicated that AcorA significantly inhibited high glucose-induced NADPH oxidase activation.

AcorA inhibited high glucose-induced PI3K-PLCγ1-PKC activation

It has been reported that PI3K-PLCγ1-PKC pathway functions as upstream pathway regulating NADPH oxidase activation.[28,29] In addition, other studies showed that PI3K-PLCγ1-PKC pathway mediates high glucose-induced collagen I production.[28] We thus evaluated the effect of AcorA on PI3K-PLCγ1-PKC signaling. HBZY-1 cells were incubated with 30 mmol/L glucose with or without 10 μmol/L AcorA pretreatment and the phosphorylation of PKC, PLCγ1, and PI3K subunit p85 was analyzed by Western blotting. As expected, high glucose enhanced the phosphorylation of two isoforms of PKC, PKCα and PKCβ1, which was decreased by pretreatment with AcorA (Figure 3A). Likewise, high glucose treatment enhanced the phosphorylation of PLCγ1 and PI3K regulatory subunit p85, which was attenuated by pretreatment with AcorA (Figure 3B). Taken together, these data indicated that AcorA inhibited high glucose induced activation of PI3K-PLCγ1-PKC pathway.

AcorA inhibited high glucose-induced ECM production

Increased synthesis and accumulation of ECM is the pathological hallmark of DN[6,7] and ROS generation contributes to ECM overproduction.[6,7] Given the fact that expression of fibronectin is present in normal mesangium and upregulated in DN, we first examined the effect of AcorA on high glucose-induced fibronectin production in MCs. HBZY-1 cells were pre-incubated with or without AcorA for 1 hour before they were exposed to 5.6 (normal glucose) or 30 mM D-glucose (high glucose) for up to 24 hours. Real time PCR revealed that high glucose upregulated the mRNA level of fibronectin by two-fold. AcorA inhibited high glucose-induced fibronectin expression in a dose-dependent manner (Figure 4A). A significant decrease of fibronectin expression was detected when HBZY-1 cells were pre-incubated with 1 μmol/L AcorA. The maximum inhibiting effect of AcorA on fibronectin production occurred at 10 μmol/L. Consistent with real time PCR data, Western blotting demonstrated that the protein level of fibronectin production induced by high glucose was also blocked by AcorA (Figure 4B). Noteworthy, the effect of 10 μmol/L AcorA on high glucose induced fibronectin production was comparable with that of 200 U/ml of cytosolic Cu/Zn superoxide dismutase (c-SOD), a potent ROS inhibitor (Figure 4), suggesting that AcorA functions as an antioxidant.

To further confirm the role of AcorA in inhibiting high glucose-induced ECM production, we next examined the expression of collagen IV, another major component of ECM. As shown in Figure 5, 30 mmol/L glucose significantly upregulated collagen IV expression. Pre-incubation of HBZY-1 cells with AcorA significantly decreased high glucose-enhanced collagen IV expression at both mRNA (Figure 5A) and protein level (Figure 5B). Likewise, the effect of AcorA was comparable with that of c-SOD. These data indicated that AcorA significantly inhibited high glucose-induced ECM production.

DISCUSSION

DN is the leading cause of end stage renal disease, and both the incidence and prevalence of DN continue to increase.[30] Currently, various treatment regimens and combinations of therapies provide only partial

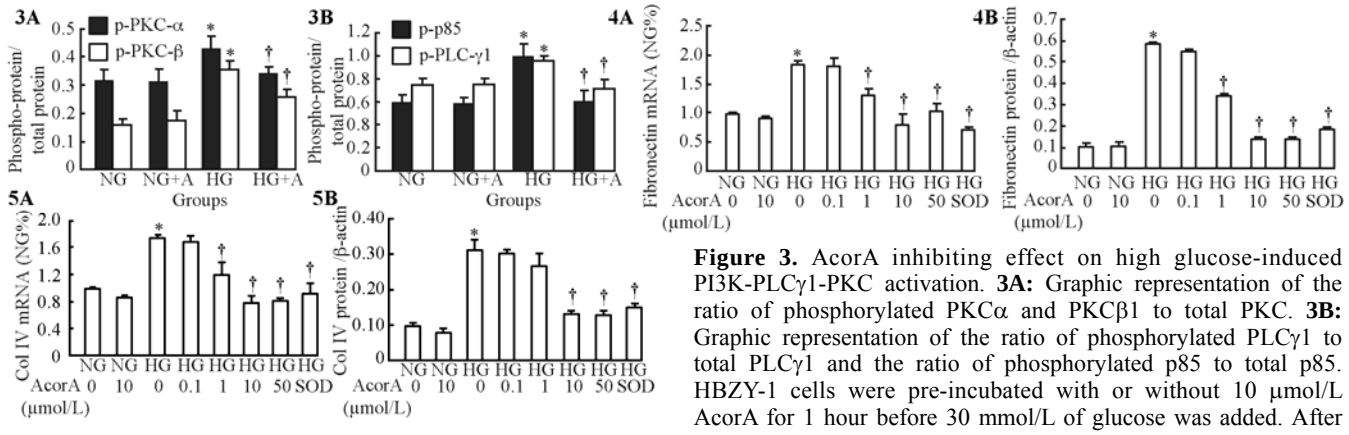

Figure 3. AcorA inhibiting effect on high glucose-induced PI3K-PLCγ1-PKC activation. **3A:** Graphic representation of the ratio of phosphorylated PKCα and PKCβ1 to total PKC. **3B:** Graphic representation of the ratio of phosphorylated PLCγ1 to total PLCγ1 and the ratio of phosphorylated p85 to total p85. HBZY-1 cells were pre-incubated with or without 10 μmol/L AcorA for 1 hour before 30 mmol/L of glucose was added. After incubation for 30 minutes, cells were harvested. The expression levels of phosphorylated and total PKCα, PKCβ1, PLCγ1, and p85 subunit of PI3K were detected by Western blotting. $^{*}P$ <0.05 versus control cells cultured in normal medium. $^{\dagger}P$ <0.05 versus 30 mmol/L glucose treated cells. Data are expressed as mean±SD of three independent experiments. NG: cells treated with 5.6 mmol/L glucose; HG: cells treated with 30 mmol/L glucose; NG+A: cells treated with 5.6 mmol/L glucose plus AcorA; HG+A: cells treated with 30 mmol/L glucose plus AcorA.

Figure 4. AcorA inhibiting effect on high glucose-induced fibronectin production at both mRNA and protein level in a dose-dependent manner. **4A:** The mRNA level of fibronectin (FN) examined by real time PCR; **4B:** the protein level of fibronectin (FN) was measured by Western blotting. HBZY-1 cells were pre-incubated with or without indicated amount of AcorA for 1 hour before 30mM of glucose was added. At 24 hours after incubation, cells were harvested. SOD: a ROS inhibitor, was used as positive control. NG: cells treated with 5.6 mmol/L glucose; HG: cells treated with 30 mmol/L glucose. $^{*}P$ <0.05 versus control cells cultured in normal medium. $^{\dagger}P$ <0.05 versus 30 mmol/L glucose treated cells. Data are expressed as mean±SD of three independent experiments.

Figure 5. AcorA inhibiting effect on high glucose induced collagen IV production in a dose-dependent manner. **5A:** The mRNA level of collagen IV (Col IV) was examined by real time PCR; **5B:** the protein level of collagen IV (Col IV) was measured by Western blotting. HBZY-1 cells were pre-incubated with or without indicated amount of AcorA for 1 hour before 30 mmol/L of glucose was added. At 24 hours after incubation, cells were harvested. SOD: a ROS inhibitor, was used as positive control. NG: cells treated with 5.6 mmol/L glucose; HG: cells treated with 30 mmol/L glucose. $^{*}P$ <0.05 versus control cells cultured in normal medium. $^{\dagger}P$ <0.05 versus 30 mmol/L glucose treated cells. Data are expressed as mean±SD of three independent experiments.

renoprotection.[31,32] Therefore new approaches are desperately needed to retard the progression of DN. In the present study, we demonstrated that AcorA, a novel spiroalkaloids isolated from *Acorus tatarinowii*, could efficiently inhibit high glucose-induced collagen IV and fibronectin production. Moreover, we explored the underlying mechanism and found that AcorA significantly blocked high glucose-induced activation of NADPH oxidase and the PI3K-PLCγ1-PKC signaling.

It is well established that high glucose-induced ROS production contributes to overproduction of ECM proteins[33,34] and different antioxidants have been shown to ameliorate high glucose-induced ECM synthesis in MCs.[11,35] Similarly, antioxidants, vitamin E and epigallocatechin-3-gallate, the most active component in green tea extracts, showed strong inhibitory effect on ECM synthesis in hepatic cirrhosis.[36,37] In agreement with these studies, we found that AcorA, at concentrations that inhibited intracellular ROS generation, effectively blocked collagen IV and fibronectin upregulation in MCs cultured under high glucose conditions. Moreover, we demonstrated that the antioxidative property of AcorA was through blocking the activation of NADPH oxidase, the most important mechanism for receptor-stimulated ROS generation.

Since high glucose-induced ROS also stimulates the ECM production in tubular cells and fibroblasts besides MCs,[34,38,39] it is plausible to assume that AcorA exerts its beneficial effect on other renal cells and contributes to the prevention of ECM accumulation in both the glomeruli and tubulariterstitium. In addition, high glucose induced ROS also leads to inflammatory cytokine production.[40] Whether AcorA could inhibit high-glucose induced inflammation is currently under investigation.

The inappropriate activation of PKC has been implicated as a putative mediator in the pathogenesis of DN based on evidence in both in vivo experimental animal models for diabetes and *in vitro* studies on cultured glomerular cells.[41-43] A variety of glomerular and MC dysfunction, such as mesangial expansion and ECM overproduction, caused by high glucose was mimicked by phorbol esters, which directly activate PKC and were abrogated by PKC inhibitors.[44-46] In addition, PKC has been shown to activate NADPH oxidase.[13] Further analysis revealed that PLCγ1 and PI3K are upstream mediators of PKC activation.[28] Therefore, we examined the effect of AcorA on the activation of PKC and found that AcorA significantly attenuated the phosphorylation of PKCα and PKCβ. Moreover, AcorA strongly reduced the phosphorylation of PLCγ1 and p86 subunit of PI3K induced by high glucose. These data suggest a role of AcorA in inhibiting PI3K-PLCγ1-PKC signaling pathway.

In conclusion, the present study demonstrated that AcorA effectively inhibited high glucose induced ECM production in cultured MCs. Although the chemical

structure of AcorA is known,[19] it is not clear at present whether MCs take up AcorA. Since excessive accumulation of ECM in the glomerular mesangium is the major pathologic feature in DN, our data suggest that AcorA might be a new therapeutic candidate for DN. Further studies are needed to determine the protective effect of AcorA against DN *in vivo*.

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