High ambient glucose induces angiotensin-independent AT-1 receptor activation, leading to increases in proliferation and extracellular matrix accumulation in MES-13 mesangial cells

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Diabetic nephropathy is associated with mesangial ECM (extracellular matrix) accumulation. We have shown that AT-1R [Ang II (angiotensin II) type I receptor] signalling induces ECM proteins via transactivation of PI3K (phosphoinositide 3-kinase) in mesangial cells. In the present study, we examined the mechanisms underlying the effect of high ambient glucose on cell proliferation and ECM expansion in a mesangial context. High glucose induced increases in PI3K activity, proliferation and ECM accumulation in mesangial cells. These effects were abrogated by losartan, an AT-1R antagonist, but not by [Sar1,Thr8]-Ang II (Sar is sarcosine), an inactive analogue of Ang II, or by a neutralizing antibody against Ang III. Overexpression of a constitutively active PI3Kα or AT-1R alone was sufficient to induce similar changes by high glucose. In contrast, overexpression of an inactive AT-1R lowered the basal levels and rendered the cells non-responsive to high glucose. Moreover, cells overexpressing wild-type AT-1R had enhanced sensitivity to acute Ang II stimulation. These cells, however, did not respond to conditioned medium obtained from mesangial cells cultured in high glucose. We further demonstrated that iAng (intracellular Ang II) can be induced by high glucose but only under certain conditions. Efficient suppression of iAng by short hairpin RNA against angiotensinogen, however, did not affect high glucose-induced effects on MES-13 cells. These results suggest that high ambient glucose induces activation of AT-1R in an Ang II-dependent manner to transactivate PI3K, resulting in proliferation and ECM accumulation in mesangial cells.

Key words: angiotensin II type I receptor (AT-1R), diabetic nephropathy, fibronectin, G-protein-coupled receptor (GPCR), phosphoinositide 3-kinase (PI3K), type IV collagen.

INTRODUCTION

The kidney is a major target organ of diabetic complications. Mesangial expansion with matrix accumulation is the pathological hallmark of diabetic nephropathy [1]. Hyperglycaemia is the primary pathogenetic factor in diabetic nephropathy [2], and numerous studies have provided evidence demonstrating that elevated glucose levels can cause an increase in the expression of various ECM (extracellular matrix) proteins in cultured MCs (mesangial cells) [3,4]. Unlike the accumulation of the ECM components, proliferation of MCs is not a typical feature of diabetic glomerulopathy [5]. However, the profile of MC proliferation may vary during the progression of the disease and may be influenced by factors in the diabetic environment. For example, in vivo studies unravelled significant MC proliferation in the early stages of diabetic nephropathy [6,7]. These studies suggest that mesangial hypercellularity precedes the increases in the ECM proteins and glomerular sclerosis. Moreover, in vitro studies have indicated that high ambient glucose stimulates MC proliferation and ECM expansion [8]. The precise molecular mechanism underlying this hyperplastic phenotype, however, has not been thoroughly explored.

Many studies have identified the RAS (renin–angiotensin system) as a key factor in the development and progression of glomerular diseases including diabetic nephropathy [9]. The octapeptide hormone Ang II (angiotensin II), the major RAS effector, activates MCs, stimulates cell proliferation and increases the synthesis of ECM components. The physiological functions of Ang II are mediated by at least two structurally and pharmacologically distinct GPCRs (G-protein-coupled receptors), AT-1R (Ang II type 1 receptor) and AT-2R. Glomerular cells primarily express AT-1R, which mediates most of the known effects of Ang II [10]. It was demonstrated that administration of ARBs (Ang II receptor blockers) slows the progression of diabetic nephropathy [11] and the development of proteinuria [12] in patients with Type 2 diabetes. This effect is specific and independent of their blood pressure lowering effect. Inhibition of the AT-1R in diabetic patients reverses endothelial dysfunction [13]. Furthermore, long-term treatment with the ARB normalizes the structure of subcutaneous small arteries of hypertensive patients with non-insulin-dependent diabetes [14]. Thus, the Ang II signalling is apparently playing a pivotal role in the pathogenesis of diabetic nephropathy. Nonetheless, the detailed mechanisms for the involvement of Ang II in the development and progression of hyperglycaemia-induced nephropathy have not been fully elucidated.

In a previous study, we demonstrated that AT-1R signalling induced ECM protein synthesis through transactivation of PI3K (phosphoinositide 3-kinase) in cultured MCs [15]. To explore how the high glucose environment affects the cross-talk between
Ang II/AT-1R and the PI3K signalling pathway, we employed an in vitro culture system by using the well-characterized mouse MC line, MES-13. With pharmacological and genetic signal modification studies, we demonstrated that ligand-independent AT-1R activation played a critical role in promoting cell proliferation and ECM protein synthesis under a high ambient glucose environment. The effects of high glucose on PI3K activity, cell proliferation and ECM synthesis were further confirmed in human primary MCs.

**EXPERIMENTAL**

**Materials**

All chemicals and reagents were obtained from Sigma–Aldrich unless stated otherwise. Anti-fibronectin (AB2033) and anti-phosphotyrosine (4G10) antibodies were obtained from Millipore. Anti-(type IV collagen) antibody (ab6586) was obtained from Abcam. Antibodies against total Akt (#9272), phospho-Akt-Thr308 (#9271) and total actin (#4968) were obtained from Cell Signaling Technology. Antibodies against phospho-Akt-Ser473 (#9275) and total actin (#4968) were obtained from Santa Cruz Biotechnology.

**Cell culture**

MES-13 mouse MCs (A.T.C.C. number CRL-1927) were seeded in a density of 1 × 10⁴ cells/ml and synchronized overnight in serum-free medium prior to start of culture. The cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with reduced (1 %, v/v) FBS (fetal bovine serum) in a humidified atmosphere containing 5 % CO₂ at 37°C for up to 96 h under normal (100 mg/dl) or high (450 mg/dl) glucose conditions. In some experiments, cells were grown up to 70 % confluence and synchronized overnight in serum-free medium prior to treatment. For isoform-selective PI3K inhibition experiments, cells were co- incubated with the following PI3K inhibitors: PI3Kα Inhibitor IV {3-(4-morpholinothio)benzene-2-yl]phenol, 2HCl; Calbiochem #528111} [16], PI3Kβ Inhibitor VI ((±)-7-methyl-2-(morpholin-4-yl)-9-(1-phenoxyethyl)-pyrido[1,2-a]-pyrimidin-4-one; Calbiochem #528113} [17] or PI3Kγ Inhibitor (5-quinoxalin-6-ylmethylene-thiazolidine-2,4-dione; Calbiochem #528106) [18]. The IC₅₀ values of these PI3K inhibitors are provided in Table 1. Primary human MCs (normal human MCs) were obtained from Lonza and maintained in MsGM medium (Lonza). Primary cells were cultured under normal and high glucose conditions as MES-13 cells. Cell proliferation was assessed using two independent methods as described in the legend to Figure 1(A).

**PI3K assays**

PI3K activity was determined with in vitro immunoprecipitation lipid kinase assay as described previously [19]. Briefly, cell lysates (0.5 mg) were immunoprecipitated with anti-phosphotyrosine antibody-coated Protein G–sepharose beads (GE Healthcare), and the beads were resuspended in assay buffer, containing 300 μM adenosine to inhibit PI4K (phosphoinositide 4-kinase) activity [20]. L-α-phosphoinositide (Avanti Polar Lipids) was used as the lipid substrate (2 μg/reaction). After incubation, the final extracted reaction mixtures were spotted on to silica gel-coated TLC plates (Whatman) and run in TLC buffer (65 % n-propanol and 0.54 M acetic acid). The results were analysed by phosphorimaging. Densitometric analysis was performed by using UN-SCAN-IT gel software for Windows (Silk Scientific).

**Western blotting**

Protein levels were measured by Western blotting cell lysates (50 μg/lane) as described in [19]. Immunoblotting of total actin was used as the loading control for fibronectin and type IV collagen expressions. Anti-fibronectin and anti-(type IV collagen) antibodies were mixed with anti-(total actin) antibody and immunoblotted simultaneously. Strength of the ECM signals was normalized against total actin signals. Phospho-Akt levels were normalized against total Akt signals. The results were visualized with chemiluminescence and analysed with UN-SCAN-IT gel software for Windows.

**Stable transfection of iSH2-p110 fusion gene**

To induce overexpression of a constitutively active PI3Kα, a 4.4 kb iSH2-p110 fusion gene of the inter-Src-homology-2 domain of the p85/p55 regulatory subunit and the p110α catalytic subunits (provided by Dr T.F. Franke, Pharmacology Faculty, Columbia University, New York, U.S.A.) was inserted into the multiple cloning site of pEGFP-N1 (where EGFP is enhanced green fluorescent protein) vector (Clontech). MES-13 cells were stably transfected with either the empty pEGFP-N1 or the vector expressing iSH2-p110. All stable transfections were performed using Lipofectamine™ 2000 (Invitrogen). Stably transfected cells were selected for neomycin resistance and individual viable cells were allowed to grow into colonies using cloning cylinders. The stably transfected cells were assayed as described above.

**Construction of the mutant AT-1R**

A vector expressing the WT (wild-type) human AT-1R cDNA (provided by Dr G. Guillemette, Department of Pharmacology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Quebec, Canada) was inserted into a pcDNA3 expression vector. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene) to generate the vector expressing constitutively active or negative AT-1R. Two oligonucleotides were constructed to introduce different mutations at Asn111 of AT-1R cDNA. The sequence of the oligonucleotide primers are: N111G (N111G-A T1, constitutively active), 5′-GCGTACAGGCCAGGAAACTGACG-3′; N111W (N111W-A T1, dominant-negative), 5′-GCGTACAGGCCAGGAAACTGACG-3′ (altered nucleotides are underlined). The site-directed mutations and integrity of the cDNAs were confirmed by DNA sequencing. Stable transfection of the constructs in MES-13 cells was performed as described above.

**RT–PCR (reverse transcription–PCR)**

Total RNA was isolated from MES-13 cells using TRIZol® reagent (Invitrogen). Reverse transcription was carried out on MES-13 cell total RNA followed by PCR using the following primer pairs: mouse GAPDH (glyceraldehyde 3-phosphate dehydrogenase) sense, 5′-TGCTACAGGCCAGGAAACTGACG-3′; mouse GAPDH

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High ambient glucose and AT-1R activation

Figure 1  High ambient glucose induces cell proliferation and ECM synthesis in MES-13 cells

Cells were cultured with reduced FBS (1 %) under normal (100 mg/dl) or high (450 mg/dl) ambient glucose for the indicated duration. (A) Cell proliferation was evaluated with two independent methods: upper panel, cells were cultured in 60-mm-diameter plastic Petri dishes. At each time point, cells were harvested using trypsin/EDTA solution and total numbers of cells were counted using a haemocytometer; lower panel, cells were cultured in a 96-well format. At each time point, MTS solution (CellTiter 96® AQueous non-radioactive cell proliferation assay, Promega Corp.) was added to each well and incubated at 37°C for 2 h. Proliferation levels were estimated based on the absorbance at 490 nm using a SPECTRAmax® GEMINI EM microplate spectrofluorimeter (Molecular Devices). Glycerol was an equimolar control for the high glucose medium. * P < 0.05 versus normal glucose. Western blotting for fibronectin and type IV collagen was performed as described in the text using anti-fibronectin (B), anti-type IV collagen (C) and anti-total actin antibodies. The histograms show the densitometric scanning results from three individual experiments. * P < 0.05.

(D) Cells were cultured with normal glucose (N), high ambient glucose (H) or equimolar glycerol-supplemented medium (Gly) for 72 h. Cellular levels of fibronectin and type IV collagen were estimated by Western blotting as described. 0 h represents quiescent status.

antisense, 5′-CTTCTGGTGGCAGTGAT-3′; human p110α sense, 5′-CCGAAAGGGTGCATTAGAG-3′; human p110α antisense, 5′-CTGAACGAGTCAACTCCAAC-3′; human AT-1R sense, 5′-GCACCTGGCTGACTTATGC-3′; human AT-1R antisense, 5′-GCAGGTGACTTTGGCTAC-3′; mouse AGT (angiotensinogen) sense, 5′-TGAATGAGGCAAGAATGTG-3′; mouse AGT antisense, 5′-AGGCTCTGAACAAATGATG-3′.

Cytosol fractionation

Cytosolic proteins were isolated using the Mitochondrial/Cytosol Fractionation kit (BioVision) following the manufacturer’s protocol. Cytosol fraction proteins (20 μg) were resolved on SDS/PAGE (10 % gels), transferred to PVDF membranes and immunoblotted with an anti-Gα11 antibody.

ELISA for Ang II

Levels of Ang II in cell lysates were measure using an enzyme immunoassay kit (Phoenix Pharmaceuticals). Cell lysates which contained 1 μg/μl cellular protein along with known concentrations of biotinylated Ang II were applied to primary antibody pre-coated 96-well ELISA plates. After the incubation, SA-HRP (streptavidin–horseradish peroxidase) solution was applied followed by TMB (tetramethylbenzidine) substrate solution. Concentrations of Ang II were estimated from absorbance at 450 nm using a spectrophotometer.

shRNA (short hairpin RNA) for AGT

AGT-specific shRNA expression pRS vectors (a mixture of four different AGT sequences) and control pRS vector (TR20003) were obtained from OriGene. Stable transfection was performed using Lipofectamine™ 2000. Stably transfected cells were selected for puromycin resistance and individual viable cells were allowed to grow into colonies using cloning cylinders. The stably transfected cells were assayed as described above.

Statistical analysis

Statistical significance of the differences between groups was analysed using paired Student’s t test or one-way ANOVA followed by a Newman–Keuls test. All statistical analyses were performed using Statistica Version 5.0 (StatSoft, Tulsa, OK,
Figure 2  High ambient glucose induces PI3K activation leading to increases in proliferation and ECM synthesis in MES-13 cells

(A) MES-13 cells were cultured with normal (N) or high (H) ambient glucose for the indicated duration and cell lysates were immunoprecipitated with an antibody specific for phosphotyrosine. PI3K activity was determined with an in vitro lipid kinase assay. (B) MES-13 cells and H9c2 cardiomyocytes were cultured with normal (N) or high (H) ambient glucose for 72 h and PI3K activities were determined as described in the Experimental section. (C) MES-13 cells were cultured with normal glucose (N), high glucose (H) or equimolar control medium (Gly) for 72 h. (D) PI3K activity was determined as described in the Experimental section. MES-13 cells were cultured with normal or high ambient glucose for 72 h with (+wort) or without (-wort) wortmannin. Cell proliferation was evaluated with two independent methods as described in Figure 1. (E) and (F) Cell lysates were subject to Western blotting using an anti-fibronectin (E) or an anti-(type IV collagen) (F) antibody. PIP, the phosphorylated end-product phosphatidylinositol 3-phosphate. 0 h represents quiescent status. The histograms show the densitometric scanning results from three individual experiments. *P < 0.05.

U.S.A.). All data are presented as the means ± S.E.M. for three different experiments unless otherwise noted in the Figure legends. A probability of P < 0.05 was considered to represent a significant difference.

RESULTS

High ambient glucose enhances proliferation and ECM synthesis in MES-13 MCs

MES-13 MCs were first seeded at 1 × 10^4 cells/ml density and synchronized overnight in serum-free medium. In order to minimize the influence of serum on the metabolism of the cells while keeping the cells in proliferative status, medium supplemented with reduced (1%, v/v) FBS was used in the experiments [21]. Cells were cultured in the reduced serum medium under normal (100 mg/dl) or high (450 mg/dl) glucose for 0 to 96 h while refreshing the medium once every 24 h. Normal glucose medium (5.6 mM) supplemented with glycerol (19.4 mM) was used as an equimolar control for the high glucose medium (25 mM). Cell proliferation was assessed with two independent methods: direct cell counting and a colorimetric assay. In both assays, high ambient glucose induced a significant increase in cell proliferation in comparison with normal glucose or equimolar control (Figure 1A). The peak induction in proliferation was seen at the 72 h time point.

In order to evaluate whether high glucose induces a fibrotic phenotype switching in MES-13 cells, we measured the cellular protein levels of fibronectin and type IV collagen by Western blotting. As shown in Figures 1(B) and 1(C), high glucose induced a significant increase in the levels of fibronectin and type IV collagen. The increases were seen as early as at the 48 h time point. The equimolar control showed no changes in the production of ECM, compared with the normal glucose group (Figure 1D). These observations are consistent with previous studies in cultured human MCs, where high glucose was shown to induce production of fibronectin and type IV collagen at both mRNA and protein levels [22,23].

High ambient glucose-enhanced PI3Kα signalling is essential for induction of cell proliferation and ECM production in MES-13 MCs

In previous studies, we have demonstrated that PI3K activation was a crucial event for the induction of ECM protein in MCs [15].
To evaluate the role of PI3K in high glucose-induced changes, we performed several sets of experiments. First, we investigated the influence of high glucose on PI3K activity. High ambient glucose induced a significant increase in phosphotyrosine-associated PI3K activity (Figure 2A). The increase in PI3K activity was seen as early as 24 h and peaked at 72 h. The induction of PI3K activity by high glucose is cell-type-dependent, since in H9c2 cells, a rat fetal cardiomyocyte cell line, high ambient glucose had no effect on PI3K under the same conditions (72 h, Figure 2B). We demonstrated further that such induction of PI3K activity was independent of osmotic pressure of the medium, as the glycerol equimolar control had a low PI3K activity similar to that seen in normal glucose control (Figure 2C). These results suggest that the effect of high glucose on PI3K activity is cell-type-dependent and that renal MCs are more susceptible to high ambient glucose than cardiomyocytes.

Secondly, MES-13 cells were treated with wortmannin (20 nM), a specific PI3K inhibitor, for 72 h under normal or high glucose conditions. In the presence of wortmannin, the effects of high glucose on cell proliferation (Figure 2D), and the levels...
of fibronectin (Figure 2E) and type IV collagen (Figure 2F) were all abolished.

Thirdly, after culturing for 72 h in normal or high ambient glucose, cell lysates were subject to an in vitro immunoprecipitation lipid kinase assay using an antibody specific for p110α, p110β or p110γ instead of using the anti-phosphotyrosine antibody. As shown in Figure 3(A), activity of p110α, but not p110β nor p110γ, was significantly induced by high glucose. The increase in PI3Kα activity was associated with activation of Akt, an important downstream signalling factor. This is shown by the significant increase in Akt phosphorylation at both critical sites (Thr308 and Ser473) as early as 48 h in high glucose (Figure 3B).

MES-13 cells were co-incubated with inhibitors (5 nM each, Calbiochem) for PI3Kα, PI3Kβ or PI3Kγ (Table 1) for 72 h under normal or high ambient glucose. Co-incubation with the PI3Kα inhibitor completely abolished high glucose-induced cell proliferation (Figure 3C) and ECM productions (Figure 3D). In contrast, neither PI3Kβ nor PI3Kγ inhibitors altered high glucose-induced effects. These results suggest that p110α is the main PI3K isoform involved in high ambient glucose-induced increases in proliferation and ECM productions in MES-13 MCs.

Fourthly, a constitutively active PI3K cDNA (iSH2-p110) was stably transfected in MES-13 cells. Expression of iSH2-p110 was confirmed by the presence of the human p110α gene by RT-PCR (Figure 4A, upper panel) and the significant increase in PI3Kα activity (Figure 4A, lower panel). The human p110α gene was not detected in WT (non-transfected) and empty vector (pEGFP-N1)-transfected cells, as the MES-13 cell line was developed from mouse kidney. Under normal glucose conditions, overexpression of the constitutively active PI3K alone was sufficient to significantly increase cell proliferation to the levels seen in control cells cultured under high glucose conditions (Figure 4B). Cell proliferation, however, was not further increased by high glucose in these PI3Kα-overexpressing cells. This suggests that cell proliferation had already reached a plateau in PI3Kα-overexpressing MES-13 cells and could not be further enhanced. Similarly, overexpression of PI3Kα alone (under normal glucose conditions) had a comparable effect on increasing the protein levels of fibronectin (Figure 4C) and type IV collagen (Figure 4D) with that of the control cells under high ambient glucose. Again, in the cells overexpressing the constitutively active PI3Kα, high glucose did not enhance the expression of these ECMs further. Moreover, under the normal glucose environment, both the protein levels of fibronectin (Figure 4C) and type IV collagen (Figure 4D) were significantly increased even at 0 h (quiescent state).
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Figure 5 AT-1R is required for high ambient glucose-induced increases in cell proliferation and ECM synthesis in MES-13 cells

Cells were cultured under normal (N) or high (H) ambient glucose for 72 h with vehicle or losartan (1 μM). (A) Cell proliferation was evaluated with two independent methods as described in Figure 1. (B) Cell lysates were subject to PIK assay as described in the Experimental section. PIP, the phosphorylated end-product phosphatidylinositol 3-phosphate. (C) and (D) Cell lysates were subject to Western blotting using antibodies against fibronectin (C), type IV collagen (D) and total actin as described in the Experimental section. The histograms show results from three individual experiments. *P < 0.05.

Taken together, data from these four sets of experiments strongly suggest that PI3Kα plays important roles in high ambient glucose-induced enhancement of cell proliferation and ECM synthesis in MES-13 MCs.

High ambient glucose-induced cell proliferation and ECM production in MES-13 MCs are mediated by activation of AT-1R

We have previously demonstrated that AT-1R signalling trans-activated PI3K to induce collagen synthesis in MCs [15]. It was not clear, however, whether AT-1R signalling is required for the effects of high glucose. To explore this, we used losartan (1 μM, Merck & Co.), an AT-1R antagonist, to block the AT-1R signalling pathway. We found that co-treatment of losartan effectively abrogated the effects of high ambient glucose on cell proliferation (Figure 5A), PI3K activities (Figure 5B) and cellular ECM productions (Figures 5C and 5D).

To demonstrate further the role of AT-1R in high ambient glucose-induced effects, we genetically manipulated AT-1R activity in MES-13 cells by stably transfecting cells with pcDNA3 (empty vector), pcDNA3 containing a constitutively active AT-1R (N111G-AT1) or a dominant-negative AT-1R (N111W-AT1) cDNA [24]. Expression of these constructs was confirmed by RT–PCR using human AT-1R gene-specific primers (Figure 6A). Again, the human AT-1R gene could not be detected in WT cells and pcDNA3-transfected cells due to the mouse origin of MES-13 cells. Under normal glucose conditions, overexpression of constitutively active AT-1R alone was sufficient to induce significant increases in PI3K activity (Figure 6B), cell proliferation (Figure 6C) and production of ECM (Figures 6D and 6E). The levels of these increases were comparable with those seen in pcDNA3-transfected cells cultured under high glucose conditions. Again, the increases induced by constitutively active AT-1R cannot be further enhanced by high glucose, suggesting they are already at maximal levels. In contrast, cells overexpressing dominant-negative AT-1R did not have high basal levels of PI3K activity, cell proliferation and production of ECM under normal glucose condition (Figures 6B–6E), nor did they respond to high glucose conditions. Interestingly, the constitutively active AT-1R-overexpressing cells only induced...
Figure 6  Constitutive activation of AT-1R alone is sufficient to induce PI3K activation, cell proliferation and ECM synthesis in MES-13 cells

MES-13 cells were stably transfected with an empty vector (pcDNA3), a constitutively active (N111G-AT1) or a negative (N111W-AT1) AT-1R cDNA. (A) Total RNA isolated from non-transfected (wild-type), empty vector-transfected (pcDNA3) or mutant AT-1R-transfected (N111G and N111W) cells were subjected to RT–PCR using primers specific for human AT-1R and mouse GAPDH. (B) The transfected cells were cultured with normal (N) or high (H) ambient glucose for 72 h. Cell lysates were subjected to PI3K assay as described in the Experimental section. PIP, the phosphorylated end-product phosphatidylinositol 3-phosphate. (C) Cell proliferation was evaluated using two independent methods as described in Figure 1. (D and E) Cell lysates were subjected to Western blotting for fibronectin (D), type IV collagen (E) and actin as described in the Experimental section. The histograms show results from three individual experiments. (F) Cells were treated under normal (N) or high (H) ambient glucose with or without co-incubation with losartan (1 μM) for 72 h. Aliquots of the cytosolic fraction (upper panel) and whole lysates (lower panel) were subjected to Western blotting using an antibody specific for Gα11. *P < 0.05.

As a member of the GPCR family, the AT-1R receptor evokes intracellular signals through G-proteins. We therefore examined whether high ambient glucose could activate G-protein through AT-1R. We found that MES-13 cells cultured under high ambient glucose for 72 h induced a significant redistribution of Gα11 subunits, a cognate G-protein of AT-1R, into the cytosol (Figure 6F). More importantly, such redistribution of G-protein was inhibited by inhibition of AT-1R with losartan (Figure 6F). Taken together, these findings suggest that the effects of the high ambient glucose on MES-13 cells are mediated via AT-1R activation.

The high ambient glucose-induced effects in MES-13 MCs are not dependent on either secreted or intracellular Ang II

As shown above, we have demonstrated the critical role of AT-1R in high glucose-induced cell proliferation and ECM synthesis in MES-13 cells. It is not clear, however, whether secreted Ang II is involved. To investigate this, we performed several experiments. First, Ang II activity was blocked with either a neutralizing antibody against angiotensin I/II (H-300; 1 μg/ml, Santa Cruz Biotechnology) or with [Sar1,Thr8]-Ang II (Sar is sarcosine) (100 nM), an analogue of and antagonist to Ang II. Neither treatment altered the effects of high ambient glucose on PI3K activity (Figure 7A), cell proliferation (Figure 7B), and protein levels of fibronectin (Figure 7C) and type IV collagen (Figure 7D) in MES-13 cells.

Secondly, we used ELISA to measure Ang II levels in conditioned culture medium from 24, 48, 72 and 96 h cultures under
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Figure 7  Inhibition of Ang II has no effects on high ambient glucose-induced effects in MES-13 cells

Cell were cultured with normal (N) or high (H) ambient glucose for 72 h with vehicle, anti-Ang II antibody (anti-Ang) or [Sar1, Thr8]-Ang II, an analogue of and antagonist to Ang II. (A) Cell lysates were subject to PI3K assay as described in the Experimental section. PIP, the phosphorylated end-product phosphatidylinositol 3-phosphate. (B) Cell proliferation was evaluated with two independent methods as described in Figure 1. (C and D) Cell lysates were subject to Western blotting for fibronectin (C) and type IV collagen (D) as described in the Experimental section. 0 h represents quiescent status. The histograms show results from three individual experiments. *P < 0.05.

high glucose conditions. Ang II concentrations in all samples were less than the detection limit (20 pM, results not shown).

Thirdly, we overexpressed WT AT-1R by stable transfection in MES-13 cells. Cells overexpressing WT AT-1R had significantly enhanced sensitivity to Ang II stimulation in activating PI3K, compared with control (pcDNA3 transfected) cells (Figure 8A). In control cells, activation of PI3K was seen only under higher dose of Ang II (10000 pM), whereas PI3K activation was obvious at a lower Ang II dose (100 pM) in WT AT-1R-overexpressing cells (Figure 8A). Under normal glucose conditions, WT AT-1R-overexpressing cells only had a moderate increase in PI3K activity (Figure 8B). Under high glucose conditions, however, these cells exhibited an exaggerated increase in PI3K activity that was effectively blocked by co-treatment with losartan (Figure 8B). Moreover, cells overexpressing WT AT-1R responded well to treatment of low doses of Ang II (100 pM) to induce Gαq11 redistribution (Figure 8C). In another experiment, WT AT-1R-transfected cells were incubated with conditioned medium prepared from 72 h high glucose culture for 0–15 min or with 100 pM Ang II for 2 min under serum-free conditions. The conditioned medium only had a marginal effect on PI3K activation and Gαq11 redistribution (Figure 8D, panel a). The effect on PI3K activity was comparable with or less than that seen in cells cultured with 1% serum (Figure 8D, panel b). In contrast, low-dose Ang II (100 pM)-treated cells (serum-free) had significantly higher PI3K activity and Gαq11 redistribution. These results indicate that the conditioned medium did not have a sufficient concentration of Ang II to stimulate AT-1R, and that AT-1R activation induced by high ambient glucose was independent of secreted Ang II.

High glucose-induced increases in iAng (intracellular Ang II) have been demonstrated [25]. In order to clarify whether iAng is involved in the high glucose-induced effects on MCs in our system, we measured Ang II contents in the cell lysates from cells cultured under various experimental conditions, as described below.
(i) Quiescent MES-13 MCs were cultured in reduced serum (1%) medium under normal or high glucose for 0, 48, 72 and 96 h. High ambient glucose induced a significant increase in cell proliferation in comparison with normal glucose or equimolar control only after 96 h of culture (Figure 9A).

(ii) Quiescent cells were cultured in medium with various concentrations of FBS (0, 0.5, 1 and 2%) under normal or high ambient glucose for 72 h. High ambient glucose induced a significant increase in iAng only under serum-free condition (Figure 9B). The induction, however, waned when 0.5% FBS was added in the culture medium and totally disappeared with 1% or higher FBS in the medium (Figure 9B).

(iii) Quiescent cells were cultured in 1% FBS under various concentrations of glucose (100, 300, 450 and 540 mg/dl). Compared with equimolar controls, there was a trend of increase in the levels of iAng with increasing concentration of glucose (Figure 9C). However, significant increases in iAng levels were observed only under the highest glucose condition tested (540 mg/dl).

Data from these three sets of experiments suggest that the effects of high ambient glucose on iAng levels in MES-13 cells are highly dependent on the culture condition and that, under the conditions we employed for our experiments (48 or 72 h, 1% FBS and 450 mg/dl glucose), the role iAng plays in high glucose-induced effects (proliferation and ECM production) in MES-13 cells is probably not significant.

To further examine whether iAng is involved in the high ambient glucose effects, MES-13 cells were stably transfected with mouse AGT-specific shRNA expression pRS vectors (OriGene). The shRNA vectors were constructed from a cocktail of four sequences (5′-CCATCTCTTTACCAACAAGGACACCT-3′, 5′-CGTCAAGGCACCTTTTGTTCAGACCT-3′, 5′-CGTTCACCTTTACCCAGAGGTAAGGT-3′ and 5′-CCTGTCTACTACGAGGATGAGGT-3′). The transfected cells showed a significant decrease in AGT gene transcription (Figure 10A, upper panel). With the AGT gene suppression, as expected, iAng levels were significantly decreased in comparison with control vector (TR20003)-transfected cells (Figure 10A, lower panel). These transfected cells were cultured under normal or high ambient glucose for 72 h. Even with complete suppression of iAng, high ambient glucose still induced significant increases in PI3K activity (Figure 10B), cell proliferation (Figure 10C) and ECM production (Figure 10D). Taken together, these data suggest the observed effects of ambient high glucose in MES-13 cells were not iAng-dependent in the current system.

**High ambient glucose induces PI3K activity, proliferation and ECM production in human primary MCs**

In order to verify the generality of the high ambient glucose-induced effects on MCs, we repeated some experiments using primary human MCs. Cells were seeded at 2 × 10^5 cells/ml density and synchronized overnight in serum-free medium. Cells were then cultured in the reduced serum (1%) medium under normal or high glucose for 48 and 72 h as described for MES-13 cells. High ambient glucose induced significant increases in PI3K activity (Figure 11A) and cell proliferation (Figure 11B) in comparison with normal glucose. In order to evaluate whether high glucose induces a fibrotic phenotype switching in the primary MCs, we measured the cellular protein levels of fibronectin and type IV collagen by Western blotting. As shown in Figures 11(C) and 11(D), high glucose induced a significant increase in the levels of fibronectin and type IV collagen.
fibronectin and type IV collagen. The increases were seen as early as the 48 h time point. Taken together, these data suggest that the high ambient glucose-induced effects on MCs are not strictly cell line-dependent and seem to be a general phenomenon for MCs.

DISCUSSION

In patients with diabetes, Ang II is believed to play a central role in the progression of renal damage not only through haemodynamic effects, but also through non-haemodynamic effects, including stimulation of growth factors, cytokines and alterations in ECM metabolism [26]. Several clinical studies have demonstrated that blockade of the RAS, either by an ACEi (angiotensin-converting enzyme inhibitor) or an ARB, reduces albuminuria and retards the progressive loss in renal function and improves survival [27,28]. These agents may have additive effects such that even greater renoprotection may be achieved when adding ARBs to maximal dosages of ACEis, suggesting that ARBs antagonized the RAS at sites where ACEi had no effect [29]. This finding is of great clinical interest because understanding the mechanisms underlying the renoprotective effects of ACEi and ARB in diabetic nephropathy and developing more selective preventive and treatment strategies is of particular importance for diabetic patients.

Various effects of high ambient glucose on MCs have been studied extensively. Among them, the two major effects which have been most widely confirmed are induction of cell proliferation and increased synthesis of ECM components. In a previous study, we demonstrated that enhanced AT-1R signalling induced collagen synthesis in MES-13 cells [15]. In the present study, we focused on how high ambient glucose affects AT-1R signalling and PI3K activity leading to induction of cell proliferation and ECM protein synthesis. We demonstrated that high ambient glucose significantly increased cell proliferation, PI3K activity and the amount of cellular type IV collagen, one of the typical ECM proteins increased in diabetic kidney, and fibronectin, the most prominently expressed ECM collagen, under normal glucose conditions. In contrast, overexpression of a constitutively activated AT-1R alone is sufficient to achieve these changes in PI3K and ECM contents under normal glucose conditions. These results suggest that the levels of AT-1R alone are not a key determining factor and that high glucose induces changes in activation of AT-1R, resulting in the observed changes in MCs.

Our studies suggest an Ang II-independent effect induced by high glucose. This conclusion is supported by the following observations: (i) ELISA failed to detect any measurable amount of Ang II (>20 pM) in conditioned medium from cells cultured under high glucose; (ii) competitive inhibition using an anti-angiotensin antibody or Ang II antagonist ([Sar1, Thr8]-Ang II) had no influence on the effects induced by high ambient glucose; and (iii) conditioned medium from cells cultured under high glucose conditions failed to induce an increase in PI3K activity when added to cells overexpressing WT AT-1R, which clearly were hypersensitive to Ang II. During the last decade, increases in the levels of iAng and secreted Ang II in culture medium have been studied extensively. Among them, the two major effects that have been most widely confirmed are induction of cell proliferation and increased synthesis of ECM components. In a previous study, we demonstrated that enhanced AT-1R signalling induced collagen synthesis in MES-13 cells [15]. In the present study, we confirmed that high ambient glucose did induce an increase in iAng levels in MES-13 cells. This was, however, highly dependent on culture conditions, including longer incubation duration (96 h), low serum concentration (0–0.5 %) and high glucose concentrations (540 mg/dl or 30 mM). Under the system we applied to our experiment (72 h, 1 % FBS, 25 mM glucose) the effects of high ambient glucose on iAng activity are unlikely to be a factor for the observed effects induced by high ambient glucose. The standard

![Figure 9](image-url)
Figure 10  High ambient glucose-induced effects in MES-13 cells are independent of iAng

Cells were stably transfected with a TR20003 control vector (TR) or a pRS vector containing the mouse AGT-specific shRNA (shAGT) sequences (sh). (A) Total RNA isolated from quiescent (0 h) or 72 h culture was subject to RT–PCR using AGT- and GAPDH-specific primers (upper panel). In another set of cultures, levels of iAng were measured by ELISA as described in the Experimental section (lower panel). TR20003- and shAGT-transfected cells were cultured with normal (N) or high (H) ambient glucose for 72 h and PI3K activities (B), cell proliferation (C) and ECM productions (D) were evaluated as described in the Experimental section. 0 h represents quiescent status. The histograms show results from three individual experiments. *P < 0.05.

method to measure Ang II levels is by ELISA. The reported iAng levels are widely different, ranging from less than 10 pg/mg of protein in rat MCs to 300 pg/mg of protein in human MCs [23,34]. Our results indicate iAng levels in mouse MES-13 cells under normal glucose condition are approx. 200–300 fmol/mg of protein, or approx. 210–310 pg/mg of protein (human Ang II, FW = 1046). In this regard, the MES-13 cells are closer to human than to rat MCs. The high glucose-induced increase in iAng levels is also modest in human MCs [23]. Despite the decent measurable iAng levels in MES-13 cells, Ang II is probably not involved in high glucose-induced effects. We were able to dramatically suppress iAng levels in MES-13 cells (from 200 to less than 10 fmol/ mg of protein) by shRNA. In the cells with such low levels of iAng, high ambient glucose still induced a significant increase in proliferation, PI3K activity and ECM protein synthesis. Hence, in our system high ambient glucose-induced enhanced activity in AT-1R seemed to be independent of its ligand.

It has been demonstrated that GPCRs can attain multiple, functionally active conformations, including those that allow constitutive signalling in the absence of ligand stimulation [37]. For the AT-1R, no naturally occurring mutations have been described that produce constitutive activation of Gαq-dependent signalling, although enhanced basal signalling has been reported in cells expressing AT-1R with engineered mutations [24]. Instead, as outlined below, some evidence exists supporting the notion that physical distortion of a cell can activate AT-1R in the absence of Ang II binding [38], which may be important to diseases such as cardiac hypertrophy. However, in the renal context, no specific condition which induces ligand-independent AT-1R activation has been identified. The findings we have shown in the present paper are the first description of ligand-independent activation of AT-1R in kidney cells. In the present study, we have demonstrated that high ambient glucose-induced AT-1R signalling transactivates the PI3K signalling pathway. During the last decade, the cross-talk

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between Ang II/AT-1R and PI3K signalling pathways has been demonstrated in cardiomyocytes, brain, neurons, vascular smooth muscle cells, and choriocarcinoma cells [39–42]. The detailed mechanisms underlying the cross-talk, however, have not been fully described. In a previous study, we delineated signalling events that mediate the effect of Ang II on collagen synthesis in MES-13 cells [15]. We demonstrated the involvement of Epac (exchange protein directly activated by cAMP), Src and EGFR (epidermal growth factor receptor)-transactivation in the cross-talk between AT-1R and PI3K. These signalling pathway factors may also be involved in the high ambient glucose-induced AT-1R/PI3K cross-talk. Further investigations into the detail of the mechanisms should be addressed in the future.

The mammalian class I PI3Ks exist in multiple isoforms, including class IA (PI3Kα, PI3Kβ and PI3Kδ) and class IB (PI3Kγ). Studies from purified recombinant proteins have shown that class IA PI3Ks respond to tyrosine kinase, whereas both PI3Kβ and PI3Kγ are sensitive to Gβγ [43]. Our laboratory has demonstrated that stimulation of β-adrenergic receptor, a GPCR, induces transactivation of PI3Kα but not other PI3K isoforms, leading to cell proliferation and ECM accumulation in MES-13 cells. Hence these results not only further challenge the common belief that PI3Kγ is the prototypical PI3K isoform activated by GPCRs [43,45], but also suggest the generality of transactivation of PI3Kα by GPCRs (in both the cardiac and nephritic context).

Emerging evidence suggests that diabetes alters the phenotype of normal non-fibroblastic kidney cells, such as MCs, tubular epithelial cells and bone marrow-derived progenitors [46]. It has been shown that cytokines, high glucose and advanced glycation end products induce profibrotic changes in the kidney cell phenotype by the processes of myofibroblast transdifferentiation and epithelial–mesenchymal transition [47]. As a result, differentiated kidney cells become reprogrammed to secrete and accumulate extracellular matrix. Among the growth factors which may be involved in the myofibroblast transdifferentiation, TGF-β (transforming growth factor-β) has been investigated most extensively. The prosclerotic cytokine TGF-β has been implicated as an important downstream mediator in the progression of the renal pathological changes occurring in diabetic patients which lead to glomerular and tubular basement membrane thickening.

Figure 11 High ambient glucose induces activation of PI3K, cell proliferation and ECM synthesis in human primary MCs

(A) Primary cells were cultured with normal (N) or high (H) ambient glucose for the indicated duration and cell lysates were immunoprecipitated with an antibody specific for phosphotyrosine. PI3K activity was determined with in vitro lipid kinase assay. (B–D) The primary MCs were cultured for the indicated duration and cell proliferation (B), fibronectin (C) and type IV collagen synthesis (D) were evaluated as described in the Experimental section. 0 h represents quiescent status. The histograms show results from three individual experiments. *P < 0.05.

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High ambient glucose leads to activation of AT-1R, which induces Gα11 translocation (black arrow) and PI3K activity. These changes may converge and induce phenotype switching of the cells which eventually leads to cell proliferation and ECM protein accumulation.

mesangial matrix expansion and glomerulosclerosis [3,48,49]. Numerous studies indicate that hyperglycaemia, as well as Ang II, induces increases in TGF-β protein levels and mRNA expression in experimental and human diabetes and cultured MCs [50–53]. Whether or not TGF-β plays a role in high-glucose-mediated changes in proliferation and ECM in a kidney context warrants further research.

In summary, we have demonstrated that high ambient glucose induces ligandless AT-1R activation, which in turn results in proliferation and hyperproduction of ECM proteins in MES-13 MCs. We have also provided evidence for the involvement of PI3Kα transactivation in the proliferative and profibrotic effect of high ambient glucose. Based on these findings, we propose a hypothetical pathway for high ambient glucose-mediated profibrotic changes in MES-13 MCs (Figure 12). Although there are other components needed to be identified in this signalling pathway, our findings nonetheless provide important information for a largely unknown mechanism of hyperglycaemia-mediated injury of MCs in diabetic nephropathy. Further study of these molecular pathways should lead to better understanding and to the identification of a new target for intervention over the conventional ACEIs or ARBs treatments for diabetic nephropathy.

**AUTHOR CONTRIBUTION**

All experiments were performed by Tram Cao, John Dahdah and Andy Tseng under the supervision of Naohiro Yano. Daïsuke Suzuki, Masayuki Endoh, Joan Stabila, Bethany McGonnigal and James Padbury proofread, discussed and corrected the manuscript prior to submission. Naohiro Yano and Yi-Tang Tseng designed experiments, analysed the data and wrote the manuscript.

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Figure 12 A hypothetical pathway for high ambient glucose-mediated effects on MES-13 MCs

High ambient glucose leads to activation of AT-1R, which induces Gα11 translocation (black arrow) and PI3K activity. These changes may converge and induce phenotype switching of the cells which eventually leads to cell proliferation and ECM protein accumulation.