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Mechanism of VEGF expression by high glucose in proximal tubule epithelial cells

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Summary

In mouse proximal tubular epithelial cells, high glucose (HG) for 24 h increases VEGF expression at the protein and the mRNA level, suggesting a transcriptional mechanism. HG-induced VEGF synthesis is prevented by captopril, an inhibitor of angiotensin-converting enzyme, and, by losartan, a specific antagonist of angiotensin type 1 receptor (AT1), suggesting that VEGF synthesis is mediated by Ang II. Synthesis of angiotensinogen (AGT), a precursor of Ang II, is induced by HG. Although renin and ACE expression is not affected, their activity is increased by HG. Ang I and Ang II synthesis is also increased by HG, and captopril prevents increased Ang II, but not Ang I, synthesis. AT1 activation is increased by with HG, and its activation is prevented by captopril and losartan. The ERK pathway is activated by HG within minutes of stimulation and lasting for up to 24h. The initial phase of ERK activation is due to HG itself and leads to AGT upregulation and the sustained phase is mediated for the most part by Ang II-activated AT1 receptor and leads to increased VEGF synthesis. In conclusion, our study shows that MCTs express an endogenous renin-angiotensin system that is activated by HG to stimulate VEGF synthesis, through activation of the ERK pathway.

Angiotensin II (Ang II) and vascular endothelial growth factor (VEGF) are important mediators of kidney injury in diabetes. VEGF expression is increased in proximal tubules of mice with type 1 diabetes. In mouse proximal tubular epithelial cells (MCT) cultured with 30 mM glucose (HG) for 24 h, VEGF expression is increased at the protein and the mRNA level, suggesting a transcriptional mechanism. HG stimulation of VEGF synthesis is prevented by captopril, an inhibitor of angiotensin-converting enzyme, and, by losartan, a specific antagonist of angiotensin type 1 receptor (AT1), suggesting that VEGF synthesis is mediated by Ang II. Synthesis of angiotensinogen (AGT), a precursor of angiotensin II, is increased in MCTs cultured in HG. Although synthesis of renin and ACE is not affected by HG, their activity is increased in the conditioned medium. Concentrations of Ang I and Ang II are also increased in conditioned medium from HG-treated MCTs and captopril prevents increased Ang II, but not Ang I, synthesis. Finally, AT1 is activated in MCTs treated with HG, and its activation is prevented by captopril and losartan. The ERK pathway is activated by HG within minutes of stimulation and lasting for up to 24h. The initial phase of ERK activation is due to HG itself and leads to AGT upregulation and the sustained phase is mediated for the most part by Ang II-activated AT1 receptor and leads to increased VEGF synthesis.

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These data show that: 1) HG increases AGT synthesis and activation of renin and ACE by MCTs, leading to local production of Ang I and Ang II. 2) Ang II activates endogenous AT1 and stimulates synthesis of VEGF. 3) HG activation of ERK starts within minutes and lasts for up to 24h. Early ERK activation is involved in AGT upregulation and sustained ERK activation, mediated via AT1, is responsible for VEGF synthesis.

In conclusion, our study shows that MCTs express an endogenous renin-angiotensin system that is activated by high glucose to stimulate the synthesis of VEGF, through activation of the ERK pathway.

Keywords

Renin-angiotensin system; VEGF; glucose; epithelial cells; kidney

Introduction

The renin-angiotensin-aldosterone system (RAS) is a major humoral system involved in the control of blood pressure and volume (Campbell, 1987). This system produces angiotensin II (Ang II) from a precursor, angiotensinogen (AGT), produced by the liver (Freeman and Rostorfer, 1972), through sequential activation of two proteases, renin and angiotensin-converting enzyme (ACE). Renin, produced in the kidney by the afferent arteriolar myoepithelial cells and released into the circulation, cleaves circulating AGT to yield a decapeptide, angiotensin I (Ang I). Ang I is cleaved in the lungs and the vascular endothelium by ACE to produce the octapeptide angiotensin II (Ang II) (Campbell, 1987). Ang II acts on target tissues by binding to two major receptors, AT1 and AT2 (Burns, 2000).

Renin is synthesized as an inactive proenzyme which is activated by cleavage by trypsin to produce the active enzyme (Persson, 2003) or without proteolysis by binding to the (pro)renin receptor (Nguyen, 2006). Unlike other proteases from its class, renin has a strict specificity for its substrate AGT (Persson, 2003). ACE, a transmembrane glycoprotein, is a nonspecific carboxypeptidase that cleaves dipeptides from a variety of substrates (Lee et al., 1971, Soffer et al., 1974). The enzyme has a greater affinity for bradykinin than for Ang I, and in addition to generating Ang II, ACE has been shown to inactivate bradykinin (Scherf et al., 1986, Kramer et al., 1990).

Since systemic Ang II levels are decreased in diabetes, local RAS has been implicated in diabetic nephropathy (Kennefick and Anderson, 1997, Lai et al., 1998, Carey and Siragy, 2003). All the components of the RAS are present within the kidney (Burns et al., 1993, Navar et al., 1996). AGT mRNA and protein are localized in proximal tubular cells, suggesting that tubular AGT provides the substrate for tubular Ang I and Ang II formation (Navar et al., 1996).

Ang II receptors belong to the G-protein-coupled receptor family and have a similar affinity for Ang II (Berry et al., 2001). AT1 is widely distributed throughout the kidney, and in vascular smooth muscle cells throughout the renal vasculature, including the afferent and efferent arterioles (Miyata et al., 1999). Angiotensin receptors are also found on proximal tubular and thick ascending limb epithelia, distal tubules, cortical collecting ducts, glomerular podocytes and macula densa cells (Miyata et al., 1999). AT2 expression is high in fetal kidney, but decreases markedly during the neonatal period (Ozono et al., 1997). In the adult kidney, it is expressed in the afferent arteriole, glomerular endothelial and mesangial cells, proximal tubular epithelial cells and interstitial cells (Miyata et al., 1999, Ozono et al., 1997).

Locally-produced Ang II contributes to hemodynamic and cellular processes involved in renal matrix expansion, proteinuria and kidney failure in type 1 diabetes (Kagami et al., 1994, Leehey

et al., 2000, Remuzzi and Bertani, 1998, Wolf et al., 1993, Wolf and Neilson, 1990). Reduction of renal hypertrophy and matrix expansion by ACE inhibitors and Ang II receptor blockers attest to the critical role of Ang II in kidney disease (Kohzuki et al., 1995, Lewis et al., 1993). Ang II effects are partly mediated by TGF β (Wolf et al., 1993, Wolf and Neilson, 1990) and other growth factors (Kagami et al., 1994). Hyperglycemia and Ang II increase VEGF synthesis in renal cells (Feliers et al., 2005, Cha et al., 2000, Pupilli et al., 1999, Kim et al., 2005), and neutralization of VEGF ameliorates proteinuria in diabetic rats (De Vriese et al., 2001, Flyvbjerg et al., 2002). Although hyperglycemia is known to activate intra-renal RAS in rats (Lansang et al., 2002), it is not known whether high glucose can activate it in the proximal tubules; whether hyperglycemia recruits RAS in proximal tubular epithelial cells to promote VEGF expression is also not known.

In view of the importance of Ang II as a mediator of hyperglycemia-associated renal injury in diabetes, we examined the role of proximal tubular epithelial cell renin-angiotensin system in the regulation of VEGF expression by high glucose.

Materials & Methods

Materials

Antibodies directed against VEGF and AGT were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against renin and ACE were from Abcam (Cambridge, MA). Conformation-specific antibodies against activated AT1 and AT2 were from Assay Designs (Ann Arbor, MI), and antibodies against AT1 and AT2 were from Alomone Labs (Jerusalem, Israel).

Cell Culture

SV40-immortalized murine proximal tubular epithelial cells (MCT) were provided by Dr. Eric Neilson (Vanderbilt University, Nashville, TN). MCTs in culture express in vivo characteristics of proximal tubular epithelial cells (Haverty et al., 1988). Cells were grown in Dulbecco's minimal essential medium (DMEM) containing 5 mM glucose and 10% FBS (Feliers et al., 2007, Sataranatarajan et al., 2008). Monolayers of MCTs were grown to 60 to 70 % confluence and serum-deprived overnight before treatment, and were barely confluent at the time of treatment.

Immunoblotting experiments were performed as previously described (Feliers et al., 2007, Sataranatarajan et al., 2008). MCTs were homogenized in lysis buffer (50 mM Tris.HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 50 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM EGTA, 0.5% Nonidet P40, and protease inhibitor mix [Sigma, St Louis, MO]). Protein concentration was measured and 10–20 μ g of whole-cell lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes and probed with various primary antibodies, and IRDye800- or IRDye700-coupled secondary antibodies were used for detection using Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE). For detection of secreted VEGF, 1 ml of conditioned medium (CM) was collected and subjected to TCA precipitation of proteins (15% TCA for 2 h at 4°C). Precipitated proteins were pelleted by centrifugation (10 min at 14,000 rpm at 4°C), and remaining TCA was extracted with ether. Pellets were suspended in 20 μ l of Laemmli sample buffer and subjected to immunoblot as described above.

Concentration of Ang I and II was measured in 100 μ l of unconcentrated CM using immunofluorescent kits from Bachem (San Carlos, CA), according to the manufacturer's instructions.

Renin and ACE activity. Renin activity was measured in 100 μ l of unconcentrated CM using the fluorometric Sensolyte Renin assay kit (Anaspec, San Jose, CA), according to the

manufacturer's instructions. ACE activity was measured in the same CM using the ACE colorimetric enzymatic assay (Alpco Diagnostics, Salem, NH).

Quantitative RT-PCR was performed as follows. RNA was extracted from treated cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR amplification of VEGF or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was performed using the Superscript One-Step RT-PCR kit from Invitrogen and the following primers:

VEGF sense (5'-ACATCTTCAAGCCGTCCTGTGTGC-3'),

VEGF antisense (5'-AAATGGCGAATCCAGTCCCACGAG-3'),

GAPDH sense (5'-CGATGCTGGCGCTGAGTAC-3'),

GAPDH antisense (5'-CGTTCAGCTCAGGGATGACC-3') (Feliers et al., 2005).

SYBR Green PCR master (Applied Biosystems, Foster City, CA) mix was added and PCR amplification was performed using a RealPlex⁴ Mastercycler (Eppendorf, Westbury, NY). Dissociation curve analysis was performed after PCR amplification to confirm the specificity of the primers. Relative mRNA expression was calculated using the $\Delta\Delta C_t$ method as reported by us previously (Sataranatarajan et al., 2007).

Statistics

Data from a minimum of 3 experiments were expressed as mean $\pm$ SEM and analyzed by ANOVA for comparison among multiple groups using Newman-Keuls post-test analysis (GraphPad Prism®); $p < 0.05$ was considered significant.

Results

High glucose increases VEGF synthesis

VEGF expression was measured in quiescent MCTs cultured in the presence of 5 mM (normal glucose, NG) or 30 mM glucose (high glucose, HG) for 24 h. HG significantly increased VEGF protein expression ($267 \pm 35\%$ of control, $p < 0.01$) in MCT lysates (Fig. 1A). Equimolar mannitol had no effect on VEGF expression. In the same cells, VEGF mRNA was also significantly increased ($433 \pm 18\%$ of control, $p < 0.001$ by ANOVA), which largely accounted for the increment in VEGF protein (Fig. 1B). These data suggest that HG stimulates VEGF synthesis through a transcriptional mechanism in MCTs and that it is not due to an osmotic effect. VEGF secretion was assessed by measuring VEGF in the CM by immunoblot. Figure 1C shows that HG ($152.1 \pm 11.6\%$ of control, $p < 0.01$), but not equimolar mannitol ($100.2 \pm 2.3\%$ of control, NS), increased VEGF secretion by MCTs.

Involvement of the renin-angiotensin system

To test the possibility that activation of an endogenous RAS mediates hyperglycemia-induced VEGF synthesis, MCTs were pretreated with captopril, a specific ACE inhibitor, or losartan, a specific AT1 antagonist, for 1 h before incubation with HG for 24 h. HG stimulation of VEGF synthesis was prevented by captopril ($110 \pm 12\%$ of control, $p < 0.01$ vs HG, NS vs NG; Fig. 2A) and by losartan ($90 \pm 16\%$ of control, $p < 0.001$ vs HG, NS vs NG; Fig. 2B). These data show that ACE and AT1 mediate the effect of HG on VEGF synthesis, suggesting involvement of RAS in MCTs.

To assess RAS recruitment by HG, we first measured AGT synthesis. Figure 3 shows that HG significantly upregulated AGT protein at 24h ($160.7 \pm 4.0\%$ of control, $p < 0.05$ vs NG) and that

this upregulation was not affected by inhibition of ACE (152.8 ± 7.6 % of control, $p < 0.05$ vs NG, NS vs HG by ANOVA).

Renin activity was measured in CM from MCTs incubated with HG for 24 h. Basal renin activity (31.1 ± 0.7 mU/mg protein) was significantly increased by HG (36.4 ± 0.4 mU/mg protein, $p < 0.05$ vs NG; Fig. 4A). ACE inhibition by captopril did not inhibit HG stimulation of renin activity (36.6 ± 0.7 mU/mg protein, NS vs HG, $p < 0.05$ vs NG; Fig. 4A). Renin activity was not affected by incubation of MCTs with equimolar mannitol (32.7 ± 0.3 mU/mg protein, $p < 0.05$ vs HG, NS vs NG; Fig. 4A). Renin protein expression was measured in the corresponding cell lysates by immunoblot (Fig. 4B). The antibody recognized a ~45 kDa band that could be pro-renin, as renin has an estimated molecular weight of 37 kDa (Nguyen, 2006). HG did not significantly modify renin expression; however, in the presence of captopril, renin expression was significantly increased by HG (214 ± 30 % of control, $p < 0.01$ vs NG; Fig. 4B).

We next measured ACE activity in the same CM. Figure 5A shows that basal ACE activity (77.2 ± 6.9 mU/mg protein) was significantly increased by HG (131.0 ± 12.8 mU/mg protein, $p < 0.01$ vs NG). Captopril prevented HG-induced stimulation of ACE activity (63.2 ± 8.8 mU/mg protein, NS vs NG, $p < 0.01$ vs HG). Similar to renin, equimolar mannitol did not stimulate ACE activity (77.3 ± 12.5 mU/mg protein, NS vs NG, $p < 0.01$ vs HG). ACE protein expression, measured in the corresponding cell lysates by immunoblot, showed that incubation of MCT with HG, with or without captopril, did not significantly modify ACE expression (Fig. 5B).

Concentration of Ang I and Ang II was measured in the same CM. Figure 6A shows that basal Ang I concentration (2.6 pg/mg protein) was significantly increased by HG (16.1 ± 1.4 pg/mg protein, $p < 0.01$ vs NG). Captopril did not affect HG stimulation of Ang I concentration (16.9 ± 1.5 pg/mg protein, $p < 0.01$ vs NG, NS vs HG). Equimolar mannitol did not increase Ang I concentration (2.0 ± 0.4 pg/mg protein, NS vs NG, $p < 0.01$ vs HG). Figure 6B shows that basal Ang II concentration (2.5 pg/mg protein) was significantly increased by HG (15.9 ± 1.2 pg/mg protein, $p < 0.01$ vs NG, NS vs HG), but not by equimolar mannitol (1.9 ± 0.5 pg/mg protein, NS vs NG, $p < 0.01$ vs HG). Captopril prevented HG-induced increase in Ang II concentration (16.9 ± 1.5 pg/mg protein, NS vs NG, $p < 0.01$ vs HG; Fig. 6B).

AT1 receptor activation

Ang II acts on cells by binding to receptors on the cell surface, AT1 and AT2. As MCTs express only the AT1, but not the AT2 receptor (data not shown), we assessed AT1 activation by immunoblot using a conformation-specific antibody that reacts only with the ligand-bound receptor (Gupta et al., 2007). Figure 7A shows that AT1 is activated by HG in MCTs in a time-dependent manner, starting at 1 h and reaching a maximum at 2–4 h (~ 200 % of control, $p < 0.01$ vs NG). Figure 7B shows that at 24 h, AT1 remained significantly activated by HG (174.1 ± 15.3 % of control, $p < 0.05$ vs NG). Captopril prevented AT1 activation at 24 h (89.8 ± 4.7 % of control, NS vs NG, $p < 0.05$ vs HG), indicating that AT1 activation at 24 h requires ACE activity and endogenous production of Ang II. Losartan, the specific AT1 antagonist, inhibited activation of AT1 by HG (88.1 ± 25.3 % of control, NS vs NG, $p < 0.05$ vs HG; Fig. 7C).

Role of extracellular-activated kinase (ERK)

We then sought to study signaling events leading to VEGF synthesis. We focused on the extracellular-regulated kinase (ERK) pathway because it is activated by HG in MCT cells (Mariappan et al., 2007), and because it is involved in stimulation of VEGF synthesis (Fukuda et al., 2002, Salem et al., 2005). Figure 8A shows that ERK is activated by HG in a time-dependent manner, starting at 5 min and reaching a maximum at 15–60 min. To examine the role of the ERK pathway in VEGF synthesis, we pre-incubated MCT cells with $10 \mu\text{M}$ of

U0126, an inhibitor of MEK1 - the kinase that activates ERK (Mariappan et al., 2007) - for 30 min prior to stimulation with HG for 24 h. Figure 8B shows that HG-induced VEGF synthesis (180.3 ± 4.1 % of control, $p < 0.01$ vs NG) was prevented by inhibition of the ERK pathway (109.3 ± 7.4 % of control, NS vs NG, $p < 0.01$ vs HG). Our data showed that AGT is upregulated by HG (Fig. 3). Inhibition of the ERK pathway by U0126 prevented upregulation of AGT by HG at 4h (135.1 ± 36.4 vs 291.0 ± 25.3 % of control, $p < 0.01$ by ANOVA). Because Ang II can also activate the ERK pathway (Duff et al., 1995) through the AT1 receptor (Ahn et al., 2004) and HG activates AT1 through generation of Ang II (Figs 6 & 7), we examined whether HG-induced ERK activation was mediated by AT1. Figure 8D shows that HG strongly activated ERK at 24 h (411.0 ± 83.1 % of control, $p < 0.01$ vs NG) and that this activation was significantly, albeit incompletely, inhibited by the AT1 antagonist (172.7 ± 19.3 % of control, $p < 0.05$ vs NG, $p < 0.01$ vs HG). Together, these data show that HG activates ERK early (minutes) and late (24h). The early activation of ERK is responsible for upregulation of AGT and the late activation is mediated for the most part via Ang II-activated AT1 receptor.

Discussion

Our study shows that incubation of MCTs with HG increases synthesis of AGT through activation of the ERK pathway, and induces activation of renin and ACE. Renin cleaves AGT to produce Ang I, which is then processed to Ang II by ACE. Ang II then binds to and activates AT1 to increase transcription of the *veg*f gene, leading to increased VEGF synthesis through activation of the ERK pathway (Fig. 9).

It is known that the kidney expresses a self-contained RAS that acts in a paracrine fashion. AGT is expressed predominantly in the proximal renal tubule (Ingelfinger et al., 1990), renin and ACE in the juxtaglomerular apparatus, and in tubular epithelial cells (Lai et al., 1998). In diabetic nephropathy, intra-renal RAS is activated and Ang II is increased (Mezzano et al., 2003) in spite of suppression of the systemic RAS (Carey and Siragy, 2003). Locally produced Ang II contributes to hemodynamic and cellular processes involved in matrix expansion, proteinuria and kidney failure in type 1 diabetes (Leehey et al., 2000). The non-hemodynamic actions of Ang II include augmented release of many growth factors, such as TGF β and Connective Tissue Growth Factor (Wolf and Ziyadeh, 2007, Ziyadeh and Wolf, 2008). Ang II can also stimulate the synthesis and release of VEGF from many tissues, including renal cells (Feliers et al., 2005, Williams, 1998, Rizkalla et al., 2003). In the kidneys, the major site of VEGF synthesis was long thought to be the podocytes (Simon et al., 1995). However, recent studies have shown that VEGF is also strongly expressed in the proximal tubular compartment in both human (Lindenmeyer et al., 2007) and mouse kidney (Feliers et al., 2005).

The existence of an endogenous RAS that is activated by HG has also been recently uncovered in the podocyte (Durvasula and Shankland, 2008), but this study did not address whether activation of local RAS was involved in VEGF synthesis. It is possible that podocyte-derived Ang II is involved in VEGF synthesis in an autocrine manner, since an AT1 antagonist prevented VEGF synthesis in podocytes in rats with type 1 diabetes (Lee et al., 2004).

Our data showed that ACE inhibition by captopril had no effect on HG-induced renin activity; however, HG augmented renin protein expression only in the presence of captopril. In humans, administration of captopril for 4 weeks resulted in increased pro-renin expression and renin activity (Sealey et al., 1981). As captopril inhibits Ang II synthesis, this upregulation of pro-renin is likely due to disruption of the negative feedback exerted by Ang II on its synthesis (Campbell, 1987). The fact that HG increases renin activity without changing pro-renin expression suggests HG acts at the level of activation. However, addition of captopril did not increase renin activity beyond that induced by HG, despite upregulation of pro-renin. This suggests that captopril fails to stimulate activation of pro-renin in MCTs. In vitro, pro-renin

can be activated by various proteases, including plasmin, cathepsin D and kallikrein (Hsueh, 1984). Kallikrein was thought to be the physiological activator of pro-renin because they colocalize in the kidney (Rohrwasser et al., 2003). Captopril could be expected to augment renin activity via kallikrein as it is also an inhibitor of kininase that degrades kallikrein. However, a role for kallikrein in renin activation was disproved by use of specific inhibitors (De Vito et al., 1996) and of knockout mice (Rohrwasser et al., 2003). Instead, it seems that in the kidney, pro-renin is activated by binding to a specific receptor (Nguyen et al., 2002), which induces a conformational change of pro-renin that allows activation without proteolytic cleavage (Nguyen, 2006). It is possible that Ang II exerts a negative feedback on pro-renin synthesis (this may not be happening as renin protein is unchanged from control in HG-treated cells) but not on the (pro)renin receptor, so that inhibition of Ang II synthesis by captopril results in upregulation of pro-renin which cannot be activated.

Our data show activation of an endogenous RAS in MCTs leads to increased extracellular synthesis of Ang II followed by autocrine action of Ang II to generate VEGF. However, our data do not rule out intracellular synthesis of Ang II, which has been demonstrated in other cell types, such as cardiomyocytes (Singh et al., 2007), mesangial cells (Vidotti et al., 2004), podocytes (Yoo et al., 2007) and vascular smooth muscle cells (Freeman and Rostorfer, 1972). The intracellular RAS (iRAS) differs from the extracellular RAS in that the conversion of Ang I to Ang II is mediated by chymase instead of ACE (Lavrentyev et al., 2007, Singh and Leehey, 2007). Intracellular Ang II translocates to the nucleus (Robertson and Khairallah, 1971), binds to chromatin-associated AT1 receptors (Booz et al., 1992, Re et al., 1984) and regulates the transcription of several genes, including those of the RAS, such as AGT and renin (Kumar et al., 2008). An important role for iRAS in hypertension-induced cardiac fibrosis (Ichihara et al., 2006) and hypertrophy (Baker et al., 2004, Mazzolai et al., 1998) has been demonstrated. Although presence of intracellular Ang II and nuclear AT1 has been demonstrated in the cortex from rat kidneys (Pendergrass et al., 2006), the role of iRAS in renal tubular injury is yet to be established.

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Figure 1. HG augments VEGF protein and mRNA expression

MCTs were incubated in normal glucose (5 mM, NG), high glucose (30 mM, HG) or high mannitol (5 mM glucose + 25 mM mannitol, HM) for 24 h. **A.** VEGF expression was measured by immunoblot, and actin was used as a loading control. The lower panel shows combined data from 4 independent experiments. ** $p < 0.01$ by ANOVA. **B.** VEGF mRNA expression was measured by qRT-PCR as described in the Methods section. Actin was used as a loading control. The lower panel shows data from 3 independent experiments. *** $p < 0.001$ by ANOVA. **C.** VEGF secretion was measured by immunoblot as described in the Methods section. Cellular actin was used as a loading control. The lower panel shows combined data from 4 independent experiments. ** $p < 0.01$ by ANOVA.

Figure 2. HG stimulation of VEGF synthesis is due to RAS activation

A. VEGF protein expression was measured by immunoblotting in MCTs incubated with NG or HG for 24 h, with or without a 1 h pre-incubation with 100 μ M captopril (HG+C). **B.** The same experiment was repeated with 100 μ M losartan (HG+L). The lower panels show data from 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ by ANOVA.

Figure 3. AGT synthesis

MCTs were incubated with NG or HG for 24 h, with or without 1 h pre-incubation with 100 μ M captopril (HG+C). AGT protein expression was measured by immunoblot, and GAPDH expression was used as a loading control. The lower panels show data from 3 independent experiments. * $p < 0.05$ by ANOVA.

Figure 4. HG activates renin

MCTs were incubated in normal glucose (NG), high glucose (HG), high glucose + captopril (HG+C) or high mannitol (HM) for 24 h. Renin activity (**A**) was measured as described in the Methods section in 100 μ l of non-concentrated CM from cells incubated as indicated. Results were derived from 4 independent experiments with duplicates for each condition. * p <0.05, NS = not significant by ANOVA. Renin expression (**B**) was measured by immunoblot on cell lysates corresponding to the CM used in **A**. GAPDH expression was used as a loading control. The lower panels show data from 4 independent experiments. * p <0.05, ** p <0.01 by ANOVA.

Figure 5. HG activates ACE

ACE activity (**A**) was measured under the same conditions as renin activity (Fig. 4), and results are expressed identically. * $p < 0.05$, NS = not significant by ANOVA. ACE protein expression (**B**) was measured by immunoblot on cell lysates corresponding to the CM used in **A**. GAPDH expression was used as a loading control. The lower panels show data from 4 independent experiments. * $p < 0.05$, ** $p < 0.01$ by ANOVA.

Figure 6. HG increases concentration of Ang I and Ang II in CM

MCTs were incubated in normal glucose (NG), high glucose (HG), high glucose + captopril (HG+C) or high mannitol (HM) for 24 h. Ang I (A) and Ang II (B) concentrations were measured as described in the Methods section in 100 μ l of non-concentrated CM. Results were derived from 4 independent experiments with duplicates for each condition. * $p < 0.05$, NS = not significant by ANOVA.

Figure 7. HG induces AT1 activity

AT1 activation was assessed by immunoblot with conformation-specific AT1 antibody (act. AT1), AT1 expression was measured with an antibody against total AT1. **A.** MCTs were incubated with HG for up to 4 h, and compared to cells incubated with NG for 4 h. **B** and **C.** Cells were incubated with HG with or without pre-incubation with 100 μ M captopril (HG+C; **B**) or 100 μ M losartan (HG+L; **B**). The lower panel shows data from 3 independent experiments. * p <0.05, ** p <0.01 by ANOVA.

Figure 8. HG activation of ERK is required for VEGF synthesis

A) MCTs were incubated with high glucose (HG) for 5 to 60 min, and ERK activation was assessed by measuring phosphorylation on Thr202/Tyr204 with a specific antibody. MCTs incubated in normal glucose (NG) for 60 min were used as control. **B)** VEGF protein expression was measured by immunoblotting in MCTs incubated with NG or HG for 24 h, with or without a 1 h pre-incubation with 10 μ M U0126 (HG+U). **C)** AGT protein expression was measured by immunoblotting in cells incubated with NG or HG for 4 h, with or without a 1 h pre-incubation with 10 μ M U0126 (HG+U). **D)** ERK activation was measured in MCTs incubated with NG or HG for 24 h, with or without a 1 h pre-incubation with 100 losartan (HG+L).

Figure 9. HG activates an endogenous RAS in MCTs to induced VEGF synthesis

We propose that glucose present in the tubular lumen is taken up by proximal tubular epithelial cells and stimulates synthesis and secretion of angiotensinogen (AGT), as well as secretion and activation of renin and angiotensin-converting enzyme (ACE). In the tubular space, AGT is converted to Ang I by renin, and Ang I to Ang II by ACE, a step inhibited by captopril. Locally formed Ang II then binds to and activates AT1 (a step blocked by losartan), which leads to stimulation of VEGF synthesis.