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

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ABSTRACT

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 24 h. 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.

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 24 h. 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.

1. 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 activatated 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

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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 TGFβ (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.

2. Materials and 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 60–70% 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 μg 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 μl of Laemmli sample buffer and subjected to immunoblot as described above.

Concentration of Ang I and II was measured in 100 μl 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 μl 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 RealPlex^4 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 three experiments were expressed as mean ± S.E.M. and analyzed by ANOVA for comparison among multiple groups using Newman–Keuls post-test analysis (GraphPad Prizm®); $p < 0.05$ was considered significant.

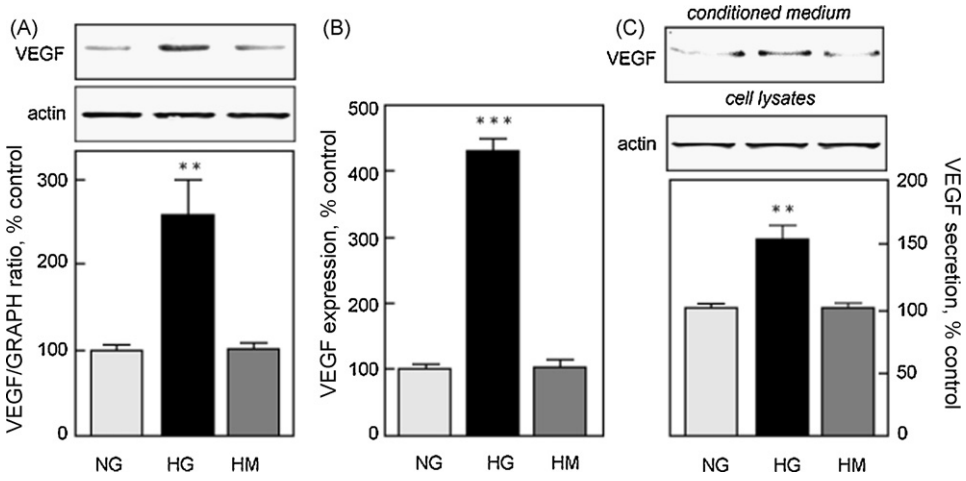

Fig. 1. HG augments VEGF protein and mRNA expression. MCTs were incubated in normal glucose (5 mM, NG), high glucose (30 mM, HG) or high mannitol (5 mM glucose + 25 mM mannitol, HM) for 24 h. (A) VEGF expression was measured by immunoblot, and actin was used as a loading control. The lower panel shows combined data from four independent experiments. $^{**}p < 0.01$ by ANOVA. (B) VEGF mRNA expression was measured by qRT-PCR as described in Section 2. Actin was used as a loading control. The lower panel shows data from three independent experiments. $^{***}p < 0.001$ by ANOVA. (C) VEGF secretion was measured by immunoblot as described in Section 2. Cellular actin was used as a loading control. The lower panel shows combined data from four independent experiments. $^{**}p < 0.01$ by ANOVA.