Cellular Signalling 22 (2010) 1849–1857

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

# Acute hyperglycemia rapidly stimulates VEGF mRNA translation in the kidney. Role of angiotensin type 2 receptor (AT2)

Robert T. Day [a], Rita de Cássia Cavaglieri [b], Hooman Tabatabaimir [a], Vasudha Mantravadi [a], Myung-Ja Lee [a], Jeffrey L. Barnes [a,c], Balakuntalam S. Kasinath [a,c], Denis Feliers [a,*]

[a] O'Brien Kidney Research Center, Department of Medicine/Nephrology, University of Texas Health Science Center, San Antonio, TX 78229, United States
[b] Medical School Hospital, University of São Paulo, São Paulo, Brazil
[c] South Texas Veterans Health Care System, San Antonio, TX 78229, United States

ARTICLE INFO

*Article history:*
Received 24 March 2010
Received in revised form 14 July 2010
Accepted 19 July 2010
Available online 24 July 2010

*Keywords:*
Kidney
Angiotensin
AT2 receptor
Vascular endothelial growth factor
Hyperglycemia
mRNA translation

ABSTRACT

Angiotensin II (Ang II) and vascular endothelial growth factor (VEGF) are important mediators of kidney injury in diabetes. Acute hyperglycemia increased synthesis of intrarenal Ang I and Ang II and resulted in activation of both Ang II receptors, AT1 and AT2, in the kidney. Losartan (specific AT1 antagonist) or PD123319 (specific AT2 antagonist) did not affect hyperglycemia but prevented activation of renal AT1 and AT2, respectively. In murine renal cortex, acute hyperglycemia increased VEGF protein but not mRNA content after 24 h, which suggested translational regulation. Blockade of AT2, but not AT1, prevented increase in VEGF synthesis by inhibiting translation of VEGF mRNA in renal cortex. Acute hyperglycemia increased VEGF expression in wild type but not in AT2 knockout mice. Binding of heterogeneous nuclear ribonucleoprotein K to VEGF mRNA, which stimulates its translation, was prevented by blockade of AT2, but not AT1. The Akt-mTOR-p70$^{S6K}$ signaling pathway, involved in the activation of mRNA translation, was activated in hyperglycemic kidneys and was blocked by the AT2 antagonist. Elongation phase is an important step of mRNA translation that is controlled by elongation factor 1A (eEF1A) and 2 (eEF2). Expression of eEF1A and activity of eEF2 was higher in kidney cortex from hyperglycemic mice and only the AT2 antagonist prevented these changes. To assess selectivity of translational control of VEGF expression, we measured expression of fibronectin (FN) and laminin β1 (lamβ1): acute hyperglycemia increased FN expression at both protein and mRNA levels, indicating transcriptional control, and did not affect the expression of lamβ1. To confirm results obtained with PD123319, we induced hyperglycemia in AT2 knockout mice and found that in the absence of AT2, translational control of VEGF expression by hyperglycemia was abolished.
Our data show that acute hyperglycemia stimulates Ang II synthesis in murine kidney cortex, this leads to AT2 activation and stimulation of VEGF mRNA translation, via the Akt-mTOR-p70$^{S6K}$ signaling pathway. Our data show that exclusive translational control of protein expression in the kidney by acute hyperglycemia is not a general phenomenon, but do not prove that it is restricted to VEGF.


## 1. Introduction

Hyperglycemia, a hallmark of type 1 diabetes, is directly responsible for most of the organ damage observed in diabetic patients, such as retinopathy and nephropathy. Although hyperglycemia can directly damage cells, some of its effects are also mediated by hormones and growth factors, the synthesis of which is increased by hyperglycemia, such as Ang II, TGFβ, connective tissue growth factor [1] and VEGF [2].

A role for VEGF in the pathogenesis of diabetic nephropathy has been established and anti-VEGF strategies such as administration of neutralizing antibodies have effectively ameliorated cardinal features of diabetic nephropathy in rodents [3]. Therefore, it is important to understand the mechanisms of VEGF regulation by hyperglycemia in the kidney.

The best documented regulation of VEGF synthesis by hyperglycemia occurs during diabetic retinopathy: in the retina of rats with type 1 diabetes, VEGF synthesis is increased in the ganglion cell layer and the inner nuclear layer [4]. Other studies have shown that upregulation of VEGF in the retina is mediated by activation of a local renin–angiotensin system [5].

The regulation of VEGF by high glucose has been studied in various kidney cells in culture. Stimulation of VEGF synthesis by high glucose has been observed in podocytes [6,7], where it is mediated by endogenous TGFβ [7], and in proximal tubule epithelial cells [6,8], where it is due to activation of a local renin angiotensin system [8].

Our previous study in proximal tubule epithelial cells has shown that Ang II rapidly stimulates VEGF synthesis, within 5 min of stimulation, through activation of the cap-dependent translation of its mRNA [9–11].

* Corresponding author. Dept. of Medicine/Nephrology, Mail Code 7882, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX, United States. Tel.: +1 210 567 4700; fax: +1 210 567 4712.
*E-mail address:* feliers@uthscsa.edu (D. Feliers).

0898-6568/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2010.07.012

However, these studies were performed in vitro using renal cells in culture without corresponding studies in animal models of hyperglycemia. In this study, we investigate whether the rapid regulation of VEGF synthesis through a translational mechanism occurs in the kidney of hyperglycemic mice and whether Ang II plays a role in that process.

## 2. Materials and methods

### 2.1. Materials

Streptozotocin, PD123,319, and antibody directed against β-actin were purchased from Sigma (Saint Louis, MO). ZD7155 was purchased from Tocris (Ellisville, MO). Conformation-specific antibodies directed against activated AT1 and AT2 were from Assay Designs (Ann Arbor, MI), and antibodies directed against AT1 and AT2 were purchased from Alomone Labs (Jerusalem, Israel). Monoclonal antibody directed against VEGF was from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were from Cell Signaling Technology (Danvers, MA).

### 2.2. Induction of hyperglycemia in mice

Induction of hyperglycemia in mice was performed as described in [12]. Four week-old C57Bl mice received daily intraperitoneal injections of streptozotocin (STZ; 50 mg/kg) for four days, followed by a fifth injection of 120 mg/kg STZ. Blood glucose was monitored using a glucometer (Ascensia Elite XL, Mishawaka, IN) every day following the last injection. Mice became hyperglycemic 4 days after the last injection. Hyperglycemic mice were injected subcutaneously with 10 mg/kg of ZD7155, 10 mg/kg of PD123319 or the equivalent volume of saline. Mice were sacrificed 24 h after injection of the antagonists, kidneys were surgically removed and cortex was dissected out for further analysis. Age- and sex-matched AT2 knockout mice (a generous gift from Dr Victor Dzau, Duke University) and their wild-type controls (129 S6/SvEvTac, Jackson Laboratories, Bar Harbor, ME) received STZ as described above and were sacrificed 24 h after onset of hyperglycemia. Animal protocols were approved by the Institutional Animal Care and Use Committee according to guidelines from the NIH.

### 2.3. Immunofluorescence histochemistry

Immunofluorescence histochemistry was performed as previously described [13]. Six-micron thick frozen kidney sections were cut using a cryostat and allowed to air dry for 45 min. The sections were fixed in ice-cold acetone for 5 min, air dried, then rehydrated in PBS, 1% BSA. After blocking with 0.5 mg/ml donkey IgG, the sections were incubated with primary antibody to AT1 and AT2 (Alomone Labs, Jerusalem, Israel), followed by FITC- or Cy-3-labeled donkey anti-rabbit IgG (Chemicon International, Inc, Temecula, CA). The sections were repetitively washed with PBS, BSA after each step. After mounting under glass coverslips the sections were viewed and photographed using an Olympus Research microscope equipped for epifluorescence.

### 2.4. Immunoblot analyses

Immunoblot analyses were performed as previously described [9,11]. Slices of kidney cortex were homogenized in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM potassium chloride, 1 mM DTT, 1 mM EDTA, 50 mM glycerophosphate pH 7.5, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM EGTA, 2 mM benzamidine, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin). Protein concentration was measured using the Biorad protein reagent (Biorad, Hercules, CA). Fifty to one hundred micrograms of kidney homogenates were separated on SDS-PAGE, transferred to nitrocellulose membranes and probed overnight at 4 °C with various primary antibodies at a 1:500 dilution, and fluorochrome-coupled secondary antibodies (Rockland, Gilbertsville, PA) were used at a 1:20,000 dilution for 15 min at room temperature for detection using Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). For the activated AT1 and AT2 immunoblots, electrophoresis was carried out under non-denaturating conditions, using a low concentration of antibodies (1:1500 dilution).

### 2.5. Polysome assay

Polysome assay was performed as previously described [9,10]. Slices of kidney cortex were homogenized in 0.4 ml of resuspension buffer containing: 10 mM Tris (pH 7.5), 250 mM KCl, 2 mM $MgCl_2$. A 10% Tween-80, 5% (w/v) deoxycholate mix was added to the lysates, which were centrifuged for 10 min at 14,000 rpm. The post-nuclear supernatants were laid on top of a 15–40% sucrose gradient, and centrifuged for 90 min at 200,000 × *g*. After ultracentrifugation, the gradients were separated into 4 fractions, and RNA was extracted from each fraction using Trizol (Invitrogen, Carlsbad, CA). Semi-quantitative RT-PCR amplification of VEGF or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was performed in polyribosomal fractions using the Superscript One-Step RT-PCR kit (Invitrogen), and the primers previously described [9].

### 2.6. Association of hnRNP K with VEGF mRNA

Association of hnRNP K with VEGF mRNA was performed as described [11,14]. Briefly, kidney cortices were homogenized in resuspension buffer and used for immunoprecipitation using anti hnRNP K antibody. After extensive wash, RNA was extracted and RT-PCR amplification of VEGF or GAPDH transcript was performed as described above. PCR products were analyzed by electrophoresis on agarose gel.

### 2.7. Phosphatase 2A (PP2A) activity

Phosphatase 2A (PP2A) activity was measured using an immunoprecipitation phosphatase assay kit from Millipore (Temecula, CA) according to the manufacturer's instruction. Equal amounts of renal cortical homogenates (250 μg) were used for immunoprecipitation for each sample. Immunoprecipitation using a non-immune serum was carried out as a negative control.

### 2.8. Statistics

Data from a minimum of three experiments were expressed as mean ± SEM and analyzed by ANOVA for comparison among multiple groups using Newman–Keuls post-test analysis (GraphPad Prizm®) and Student's *t*-test was used for comparison between two groups; $p<0.05$ was considered significant.

## 3. Results

### 3.1. Induction of hyperglycemia in mice and effect of angiotensin receptors antagonists

We used streptozotocin (STZ) to induce hyperglycemia in mice, as described in the Materials and methods section. Blood glucose was monitored daily: mice became hyperglycemic (>250 mg/dl) 4 days after the last STZ injection. At that point, angiotensin receptor antagonists (ZD7155, a long-acting AT1 antagonist [15] or PD123319, an AT2 antagonist) or vehicle were injected subcutaneously and mice were sacrificed 24 h after injection. We chose to study an early time-point when mechanisms of rapid control of protein synthesis, such as activation of mRNA translation, are activated. STZ-injected mice became significantly hyperglycemic (299.2 ± 15.5 vs 154.1 ± 5.3 mg/dl, $p<0.01$), and injection of AT1 or AT2 antagonists,