Aldosterone Stimulates Fibronectin Synthesis in Renal Fibroblasts under Hyperglycemic Conditions: Role of JNK and AP1

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ABSTRACT

The mineralocorticoid hormone aldosterone plays an independent role as a mediator of kidney injury and progression of chronic kidney disease. Clinical studies confirmed an independent effect of aldosterone in the progression of human kidney disease. Aldosterone activates various molecules that result in the abnormal accumulation of extracellular matrix proteins that lead to kidney fibrosis. However, the cell mechanisms that lead to aldosterone-dependent matrix synthesis are poorly understood. In this study we attempt to identify the molecular mechanisms that mediate aldosterone-dependent synthesis of interstitial matrix proteins in kidney fibroblasts. We find that aldosterone significantly stimulates fibronectin synthesis and that this effect is particularly enhanced in cells exposed to high glucose concentrations. Aldosterone-dependent FN synthesis is mediated through activation of JNK2, ERK1/2 and PKD signaling pathways. JNK2 signaling induces the phosphorylation of the AP1 transcription factor c-jun, which translocate to the nucleus of NRK-49F cells and forms a nuclear complex with the mineralocorticoid receptor. We propose that the above mechanism may play an important role in aldosterone-dependent progression of diabetic nephropathy.

BACKGROUND

Aldosterone, a mineralocorticoid hormone that is primarily synthesized in the adrenal gland, is a major regulator of extracellular fluid volume and sodium and potassium balance (1). However, several studies over the past decade have shown, that aldosterone plays an independent role as a mediator of kidney injury and progression of chronic kidney disease (2). Aldosterone is synthesized in the kidney (3) and the aldosterone receptor is present in mesangial cells, renal fibroblasts and in tubular epithelial cells (4). An independent role of aldosterone in progression of chronic kidney disease (CKD) was first shown in a 5/6 nephrectomy rat model, where intravenous infusion of aldosterone diminished the renal protective effect of ACE-inhibitors (5). Clinical studies confirmed an independent effect of aldosterone in human renal disease progression despite ACE-Inhibitor or ARB treatment (4). A role of aldosterone in progression of diabetic nephropathy was shown in a diabetic rat model (6) and in hypertensive type I diabetic patients (7).

Progression of CKD correlates with increase in interstitial fibrosis in the kidney (8). Aldosterone activates various molecules that result in the abnormal accumulation of extracellular matrix proteins that lead to kidney fibrosis (9) The action of aldosterone is mediated through a ligand-activated transcription factor, the mineralocorticoid receptor (MR) (10). The classic mechanism for the aldosterone-mediated MR signaling pathway is through regulation of transcription (11). However, no unique MR response element (MRE) for MR-induced genes has been identified to date, indicating that additional co-factors might play an important role in MR-dependent gene transcription (11). Despite increasing evidence that aldosterone stimulates fibrosis in the kidney, the cell mechanisms of aldosterone-dependent fibrogenesis are still enigmatic.

METHODS

DMEM with high glucose (4.5 g/L D-glucose) and low glucose (1.0 g/L D-glucose) concentrations were used to test the aldosterone-dependence of FN synthesis. NRK-49F cells (ATCC, Manassas) were treated with aldosterone (Sigma) at 5 nmol/L or 20 nmol/L concentrations. NRK-49F cells were incubated with serum-free medium (0.5% BSA) or medium containing 1.0% FCS in the presence or absence of aldosterone. Total RNA was isolated using an RNeasy kit (QIAGEN) following on-column DNase digestion. First-strand cDNA synthesis was accomplished using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Amplification and detection of specific products were carried out on an ABI 7300 real-time cycler (ABI). Fluorescent signals were normalized to an internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The relative expression level between treatments was then calculated using the following equation: relative gene expression = $2^{-(\Delta Ct sample - \Delta ct control)}$. Cells were treated with aldosterone for either 48 hours (for fibronectin abundance) and pre-treated for 1 hour with Eplerenone for the inhibitor experiments. For the ERK inhibitor studies NRK 49f cells were treated for 1 hour with 10 μM UO126 (Promega). JNK inhibitor was used at a concentration of 10 μM (Calbiochem) for 60 minutes prior to aldosterone stimulation. The PKD-specific inhibitor CRT was a generous gift from Dr Sushovan Guha, MD Anderson Cancer Center. After being transferred onto a nitrocellulose membrane, samples hybridized overnight with rabbit anti-fibronectin antibody, rabbit anti-pERK, anti-PKD/PKCμ(Ser744/748) antibody, anti-c-jun and anti-p-c-jun antibodies, anti-JNK2 antibody and anti-MCR-antibody or mouse monoclonal anti-β-actin. Cells were incubated with primary antibodies followed by incubation with fluorescein-labeled anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA). Actin filaments were stained with Rhodamine Phalloidin (Invitrogen, Eugene, Oregon) for 30 min. Slides were examined using a Zeiss Axiophot inverted microscope, and images were obtained and processed using the MagnaFire 2.1C Software (Olympus American, Melville, NY).

RESULTS

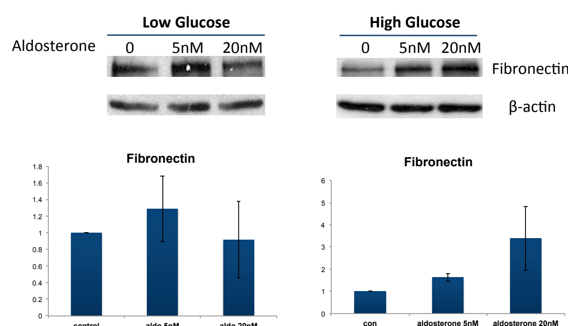

Figure 1. Aldosterone treatment increased fibronectin (FN) abundance in kidney fibroblasts (NRK 49f cells). Exposure to low concentration glucose (1 g/L) culture medium, containing 5 nM aldosterone, caused only a small increase in FN abundance. NRK 49f cells grown in high concentration (4.5 g/L) glucose medium showed a near two-fold increase in FN levels when treated with 5 nM aldosterone and a 3.5 fold increase in FN with 20 nM aldosterone treatment.

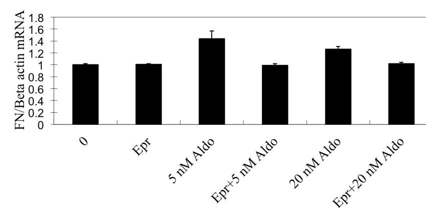

Figure 2. Aldosterone treatment increased fibronectin mRNA levels in NRK 49f cells grown in high glucose medium. FN mRNA levels increased by 50% following 5 nM aldosterone treatment and increased by 30% following 20 nM aldosterone treatment. Aldosterone-dependent fibronectin transcription was inhibited following pre-treatment of NRK 49f cells with the mineralocorticoid receptor inhibitor Eplerenone.

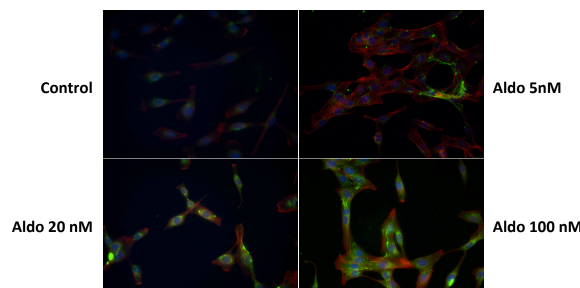

Figure 3. Immunofluorescence studies in kidney fibroblasts using GFP-labeled anti-fibronectin antibody (green), showed marked increases in cytoplasmic and extracellular FN following treatment with 20 and 100 nM aldosterone under high glucose conditions. Red=phalloidin; Blue=dapi nuclear stain.

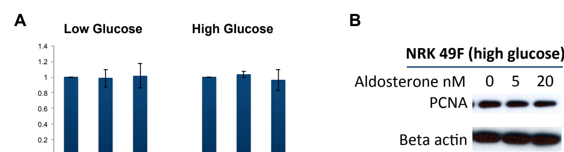

Figure 4. (**A**) Cell viability and proliferation following aldosterone treatment of NRK 49f cells was assessed using MTT assay (**A**) and PCNA western blot (**B**). Aldosterone treatment with either 5 or 20 nM did not significantly decrease cell viability in NRK 49f cells (**A**). PCNA abundance did not increase following aldosterone treatment of NRK 49f cells (**B**).

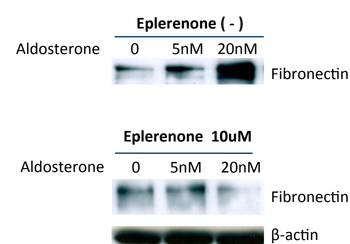

Figure 5. To examine the role of the mineralocorticoid receptor (MCR) in aldosterone-dependent fibronectin synthesis, we treated NRK 49f cells with the MCR-specific inhibitor Eplerenone prior to aldosterone exposure. 10 uM Eplerenone decreased fibronectin abundance significantly in both 5 and 20 nM aldosterone treatment groups.

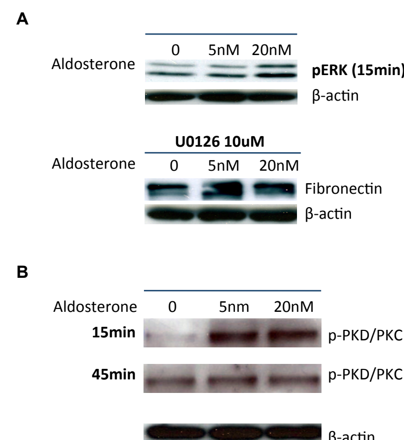

Figure 6. (**A**) Aldosterone treatment stimulated ERK signaling levels in NRK 49f cells within 15 minutes of exposure. 20 nM aldosterone induced a stronger pERK signal than 5 nM at this time point. Pre-treatment of NRK 49f cells with the highly selective MEK inhibitor UO126 decreased aldosterone-dependent fibronectin synthesis by 50% in the 20 nM treatment group.
(**B**) Aldosterone treatment with 5 and 20 nM for 15 minutes significantly stimulated PKD/PKCu signaling in NRK 49f cells. After 45 minutes p-PKD/PKCu signaling levels had returned to baseline levels.

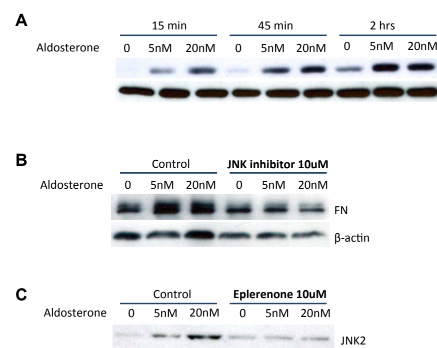

Figure 7. (**A**) Aldosterone treatment of NRK 49f cells stimulated the JNK2 signaling pathway with the greatest activity seen at 45 and 120 minutes. Treatment with 20 nM aldosterone stimulated JNK2 at both 15 and 45 minutes stronger than 5 nM treatment. (**B**) Pretreatment with JNK inhibitor (10 μM) significantly decreased aldosterone-dependent FN synthesis. (**C**) Pretreatment with mineralocorticoid receptor-specific inhibitor Eplerenone significantly decreased aldosterone-dependent JNK2 activation.

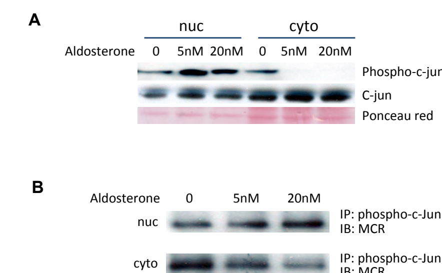

Figure 8. (**A**) Aldosterone-dependent total c-jun and phospho-c-jun abundance was measured in nuclear and cytoplasmic extracts of NRK 49f cells after treatment with 5 and 20 nM aldosterone. Aldosterone treatment increased phospho-c-jun nuclear abundance in NRK 49f cells, while total c-jun increased in cytoplasmic fractions. (**B**) Immune precipitation assays in nuclear and cytoplasmic extracts of NRK 49f cells using phospho-c-jun as ligand, showed aldosterone-dependent increase in mineralocorticoid receptor abundance in nuclear extract. The cytoplasmic extract showed decreased MCR abundance.

CONCLUSIONS

Our study aimed to examine the signaling pathways and transcription factors involved in aldosterone-dependent fibronectin expression in a kidney fibroblast cell line (NRK 49f cells). Our results show that aldosterone increased both fibronectin abundance and message at 5 and 20 nM concentrations. Aldosterone-dependent fibronectin synthesis was mediated through the mineralocorticoid receptor and dramatically augmented by exposure to high glucose medium concentrations. Surprisingly aldosterone treatment did not stimulate fibroblast proliferation or TGFβ-dependent signaling pathways. The signaling pathways stimulated by aldosterone in NRK 49f cells were pERK and p-PKD/PKCμ. Moreover, aldosterone-dependent fibronectin synthesis was dependent on JNK2 signaling. Abundance of the AP1 family transcription factor c-jun increased following aldosterone treatment and translocation of phosphorylated c-jun together with the mineralocorticoid receptor might be an important step in aldosterone-dependent fibronectin synthesis.

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