Aldosterone induces CTGF in mesangial cells by activation of the glucocorticoid receptor

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Abstract

Background. Aldosterone contributes substantially to cardiac and renal injury by acting on target cells not involved in the regulation of salt and water balance. The profibrotic protein connective tissue growth factor (CTGF) has been identified as one of the target proteins of aldosterone. However, the molecular mechanisms of aldosterone-mediated CTGF induction have not been characterized.

Methods. Mesangial cells were treated with aldosterone or dexamethasone. CTGF expression was characterized at the mRNA and protein level. Translocation of the glucocorticoid receptor (GR) was detected by immunocytochemistry and by Western blotting.

Results. Aldosterone and dexamethasone induced CTGF at the mRNA and protein level in a time- and concentration-dependent manner. Specific antagonists of the mineralocorticoid receptor, spironolactone, canrenoate or eplerenone, did not inhibit CTGF induction. However, inhibition of the GR by RU486 prevented dexamethasone-as well as aldosterone-induced CTGF expression, indicating the importance of the GR in aldosterone-mediated regulation of CTGF. This notion was confirmed by translocation of the GR to the nucleus upon stimulation with aldosterone.

Conclusions. CTGF is a functional target of aldosterone in mesangial cells, but aldosterone-induced CTGF gene expression is not directly mediated by the mineralocorticoid receptor.

Keywords: Aldosterone; connective tissue growth factor; dexamethasone; glucocorticoid receptor; mesangial cells; mineralocorticoid receptor

Introduction

Pathological activation of the renin-angiotensin-aldosterone system leads to end-organ damage which is at least in part independent of haemodynamic actions of angiotensin II or aldosterone. By activation of AT1 and/or AT2 receptors, angiotensin II can induce renal or cardiac injury including glomerulosclerosis and cardiac hypertrophy [1,2]. Activation of AT1 by angiotensin II regulates the expression of profibrotic factors such as connective tissue growth factor (CTGF) [3] or the excessive synthesis of extracellular matrix. Furthermore, it has become apparent that aldosterone substantially contributes to organ injury by acting on non-classical mineralocorticoid target cells [4]. Beneficial effects of mineralocorticoid receptor (MR) antagonists were observed in patients with heart failure and other cardiovascular diseases, suggesting direct effects of aldosterone on the expression of profibrotic proteins [5]. Consistent with this concept, in vivo experiments showed upregulation of CTGF in aldosterone-mediated cardiac hypertrophy in mice [6]. Aldosterone was also implicated in the progression of human and experimental diabetic nephropathy, which was ameliorated by treatment with spironolactone [7,8].

In vitro, regulation of CTGF by corticosteroids is far from understood. First observed in fibroblasts [9] upregulation of CTGF by glucocorticoids was confirmed in several other cell types including chondrocytes and osteoblasts, but also renal cells, mesangial and proximal tubular cells [10–13]. In mice, induction of CTGF by dexamethasone seems to be dependent on the genetic background of the animals [10]. CTGF belongs to the group of early response genes that are rapidly regulated at the transcriptional level, as shown for various stimuli such as TGF-β or bioactive lipids (e.g. [14,15]). Transcriptional as well as post-transcriptional mechanisms have been proposed for dexamethasone-induced expression of CTGF, but no unifying mechanisms have been established yet [11]. Recent data indicate that aldosterone can induce
CTGF in vivo. Aldosterone-mediated upregulation of CTGF was detected in mouse mesangial cells and tubular epithelial cells within 24 h [16]. It was sensitive to treatment with an antagonist of the MR, spironolactone. These findings have recently been confirmed in vascular smooth muscle cells [17]. In myocytes, aldosterone-induced upregulation of CTGF was only observed in cells expressing the serum and glucocorticoid-induced kinase (SGK) indicative of a role of this aldosterone-induced kinase in CTGF induction [6]. Formerly, tubular epithelial cells were considered to be the sole target cells of aldosterone in the kidney. More recently, expression of the MR has also been characterized in glomerular podocytes [18] and mesangial cells [19–21]. Stimulation of mesangial cells with aldosterone enhanced proliferation, which was attributed to an early activation of mitogen-activated kinases [19,20] and increased synthesis of extracellular matrix molecules via a SMAD2 dependent pathway [21]. In a recent study, we observed MR-dependent upregulation of the chemotactic protein osteopontin in mesangial cells, linking MR activation to the regulation of gene expression in these cells [22]. Furthermore, mesangial cells were shown to synthesize aldosterone [23]. Therefore, in addition to the systemic renin-angiotensin-aldosterone system, mesangial cells may contribute to increased local aldosterone concentrations and aggravate aldosterone-mediated glomerular injury.

The molecular actions of aldosterone are complex. It binds with high affinity to intracellular MRs, but has also been shown to bind to glucocorticoid receptors (GR) albeit with much lower affinity [1,24,25]. Furthermore, rapid non-genomic effects are observed in cells treated with aldosterone [26,27]. Aldosterone receptor antagonists are commonly used to differentiate between MR and GR actions. Spironolactone and its metabolite canrenoate bind with high affinity to the MR, but also interact with other steroid receptors, progesterone and androgen receptors. These interactions were implicated in unwanted effects observed when these drugs are used therapeutically [28]. At micromolar concentrations, spironolactone even interacts with the GR. Eplerenone, although less potent as antagonist at the MR, is more specific and does not interact with other steroid receptors.

Direct target genes of aldosterone are rare, and even though CTGF has been shown to be a functional target of aldosterone, it has not yet been investigated whether CTGF is induced by the MR. It was thus the aim of the present study to analyse the molecular mechanism of the induction of CTGF in mesangial cells by aldosterone.

Materials and methods

Materials

Aldosterone, dexamethasone, canrenonate, RU486 (mifepristone) and spironolactone were obtained from Sigma, Munich, Germany. Eplerenone was kindly provided by Pfizer, Karlsruhe, Germany. A protease inhibitor cocktail was obtained from Roche Diagnostics, Mannheim, Germany.

Cell culture

Rat mesangial cells were isolated from male Sprague Dawley rats as described previously [29]. The cells exhibited the typical stellate morphology and were positive for Thy-1, desmin and α-actin and negative for factor VIII. Rat mesangial cells also expressed mineralocorticoid receptor antagonists as shown recently [22]. Cells were grown in DMEM supplemented with 2 mM l-glutamine, 5 μg/ml insulin, 4.5 g/l glucose, 100 U/ml penicillin and 100 μg/ml streptomycin containing 10% (v/v) FCS. Mesangial cells were used between passages 10 and 20. For the experiments, mesangial cells were seeded in medium with 10% (v/v) FCS and then growth-arrested overnight in medium without serum.

Western blot analysis

CTGF was detected in cellular homogenates by Western blot analysis. The following antibodies were used: goat polyclonal anti-CTGF (SC 14939, anti-goat IgG conjugated to horseradish-peroxidase, mouse anti-glucocorticoid receptor (Santa Cruz, Heidelberg, Germany); mouse anti-tubulin antibody E7, developed by M. Klymkowsky (Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences Iowa City, IA, USA); peroxidase-conjugated anti-mouse secondary antibody (Amersham Biosciences, Freiburg, Germany); anti-vinculin (Sigma). Protein–antibody complexes were visualized by the enhanced chemiluminescence detection system (ECL, Amersham Biosciences). To confirm equal loading and blotting, the blots were stained with amido black or reprobed with vinculin or tubulin. For quantification purposes, blots were quantified using a luminescent imager (LAS-1000 Image Analyser, Fujifilm, Berlin, Germany) and Aida 2.1 image analysis software (Raytest, Berlin, Germany).

Preparation of nuclear extracts

Mesangial cells were lysed in 10 mM HEPES, pH 7.9, containing 1% NP-40, 0.1 mM EGTA, 10 mM KCl, 1 mM DTT and a protease inhibitor cocktail for 10 min in the cold. After centrifugation, nuclei were lysed with 10 mM HEPES containing 10% glycerol, 0.3 M NaCl, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail for 20 min. After centrifugation, the nuclear extract was analysed for glucocorticoid receptor protein by Western blotting.

Northern blot analysis

CTGF mRNA expression was detected by northern blot analysis using standard techniques. Hybridization was performed with cDNA probes labelled with [32P]dCTP using the NonaPrimer kit from Appligene, Heidelberg, Germany. A cDNA specific for CTGF was kindly provided by N. Wahab, London, UK. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) probe was obtained with a 500 bp reverse transcribed fragment. DNA/RNA hybrids were detected by autoradiography using a Kodak X-Omat AR film.
Induction of CTGF by aldosterone and dexamethasone in mesangial cells

Mesangial cells were treated with aldosterone (10^{-6} M) for up to 24 h. Cell-associated CTGF was detected by Western blot analysis. CTGF protein was upregulated within 3–4 h and remained elevated over the time course of 24 h (Figure 1A). When the cells were treated with dexamethasone (10^{-6} M), a comparable increase in CTGF protein was obtained after 24 h (Figure 1B). As CTGF is a rather unstable protein with a half-life of ~2 h as determined in the presence of cycloheximide (data not shown), continuous stimulation of CTGF biosynthesis is necessary to sustain elevated CTGF levels. The concentration-dependence of CTGF induction was assessed at 3 h. The maximal stimulation of CTGF induction by high concentrations of aldosterone or dexamethasone was comparable, ~2-fold as shown in Figure 1C. At low concentrations (10^{-8} M), however, only dexamethasone induced a statistically significant expression of CTGF, while higher concentrations of aldosterone (10^{-7} to 2 \times 10^{-7} M) were needed to obtain a comparable stimulation (Figure 1D). The rapid induction of CTGF was confirmed at the mRNA level, showing increasing CTGF mRNA levels within 1–3 h, which remained elevated over 24 h (Figure 1E). Elevated mRNA levels, which persist for hours, may be due to message...

Immunocytochemistry

Mesangial cells were fixed with 3.5% paraformaldehyde in PBS for 10 min and permeabilized in 0.2% Triton X-100 in PBS. The anti-glucocorticoid receptor antibody was diluted 1:200, the anti-mouse secondary antibody coupled to Alexa Fluor 488 ( Molecular Probes, Leiden, The Netherlands) was used at 1:500. Images were obtained using a Leica microscope and processed using Adobe Photoshop CS.

Statistical analysis

All data are presented as means ± SD. Statistical analysis was done using analysis of variance (ANOVA) with the Tukey–Kramer multiple comparisons test. A value of \( P < 0.05 \) was considered statistically significant.

Results

Induction of CTGF by aldosterone and dexamethasone in mesangial cells

Mesangial cells were treated with aldosterone (10^{-6} M) for up to 24 h. Cell-associated CTGF was detected by Western blot analysis. CTGF protein levels in non-stimulated cells (Co) were set to 1. Data are means ± SD of \( n = 3 \) (dexamethasone) experiments; \( ^* P < 0.01 \) compared with control. (C) Mesangial cells were stimulated with aldosterone (10^{-6} M) for 24 h. CTGF was detected by Western blot analysis. The blots were reprobed with tubulin to confirm equal loading and blotting. (D) Experiments were performed as in C with aldosterone at 10^{-8}, 2 \times 10^{-7}, or 10^{-6} M and dexamethasone at 10^{-5} or 2 \times 10^{-7} M. The expression of CTGF in non-stimulated cells (Co) was set to 1. Data are means ± SD of five or more experiments; \( ^* P < 0.01 \); \( ^{**} P < 0.001 \). (E) Mesangial cells were treated with aldosterone (10^{-6} M) or dexamethasone (10^{-7} M) for the times indicated. CTGF mRNA levels were determined by Northern blot analysis. At each time point, expression of CTGF mRNA in the presence of corticosteroid was related to the expression of CTGF in cells cultured without corticosteroid. CTGF levels of non-stimulated cells were set to 1. Error bars indicate half range of two experiments.
stabilization. However, when mesangial cells were first treated with dexamethasone (10^{-6} M) for 16 h and then with actinomycin D to interfere with ongoing transcription, CTGF mRNA levels declined rapidly (t_{1/2} \sim 2.5 h, data not shown). In order to characterize the molecular mechanism of aldosterone-mediated CTGF upregulation, all further analyses were performed after stimulation for 3–4 h. As the induction of CTGF was not significantly different between 3 and 4 h, the data obtained at these time points were compiled.

Interference of receptor antagonists with aldosterone- and dexamethasone-mediated induction of CTGF

The expression of mineralocorticoid receptors (MR) has been described in mesangial cells. Therefore, we investigated the role of MR in CTGF induction using specific antagonists, spironolactone, canrenoate and eplerenone. A 10-fold excess of spironolactone (10^{-6} M) over aldosterone (10^{-7} M) did not inhibit the aldosterone-mediated induction of CTGF (Figure 2A/B). When spironolactone was used at a concentration of 10^{-5} M (100-fold excess), CTGF induction was completely suppressed. However, at this concentration, spironolactone also suppressed the basal expression of CTGF as well as the dexamethasone-induced expression of CTGF (Figure 2A/B). Therefore, at 10^{-5} M, spironolactone showed non-specific effects in mesangial cells.

Preincubation of mesangial cells with either canrenoate or eplerenone in concentrations up to 10^{-5} M did not affect the basal or dexamethasone-induced CTGF expression (Figure 2C). However, aldosterone-mediated upregulation was not affected either. These data argued against direct genomic effects exerted by the MR on CTGF expression.

Next, we investigated the role of the glucocorticoid receptor (GR) in the regulation of CTGF expression. Treatment of mesangial cells with RU486 (10^{-6} M), a specific antagonist of the GR and progesterone receptor, did not affect the basal expression of CTGF (Figure 3A and B). As expected, preincubation of mesangial cells with RU486 prevented the dexamethasone-mediated upregulation of CTGF. In addition, RU486 also inhibited aldosterone-mediated CTGF induction, indicative of a role for the GR in aldosterone-mediated action in mesangial cells.

Translocation of glucocorticoid receptors to the nucleus

Upon activation, the GR is translocated from the cytosol to the nucleus. Therefore, we investigated whether aldosterone was able to translocate the GR. Compared with control cells, accumulation of GR immunoreactivity was observed in the nuclei of mesangial cells after stimulation with dexamethasone (10^{-6} M) or aldosterone (10^{-6} M) for 30 min (Figure 4A). To quantify GR protein in the nuclei, Western blot analyses were performed with nuclear extracts. A significant increase in nuclear GR was detectable within 10 min after stimulation of the cells with either dexamethasone or aldosterone (Figure 4B and C). In line with the immunocytochemical analysis, GR translocation was also detectable after 30 min, showing a direct effect of aldosterone on the translocation of the glucocorticoid receptor.

Discussion

CTGF is recognized as a profibrotic protein and plays a critical role in renal pathology. Upregulation of CTGF has also been linked to aldosterone-mediated renal injury [1]. Our studies provide evidence that CTGF is a functional target of aldosterone, but not a direct target of the MR in mesangial cells.

Treatment of mesangial cells with either aldosterone or dexamethasone induced CTGF at the mRNA and protein level. The kinetics and the magnitude of CTGF induction by high concentrations of the corticosteroids were comparable, reaching a maximal stimulation of about ~3-fold. The effect was long lasting and elevated levels of CTGF were still detectable after 24 h, indicative of a continuous stimulation of CTGF protein.
synthesis. Dexamethasone was more potent than aldosterone, and higher than physiological concentrations of aldosterone were needed to induce CTGF. The concentration-dependence thus argued against the MR as mediator of the aldosterone effect, because aldosterone activates its cognate receptor in the nanomolar range, whereas 100-fold higher concentrations of aldosterone are necessary to activate the GR [24].

To further clarify the mechanism of aldosterone-induced CTGF induction, we used different receptor antagonists. Spironolactone is commonly used as specific antagonist of the aldosterone receptor. In animal models of renal injury (e.g. [16]), and also in patients with diabetic nephropathy [30] spironolactone has been shown to provide beneficial effects, which were not attributable to its haemodynamic effects. In our study, high concentrations of spironolactone inhibited dexamethasone- or aldosterone-stimulated CTGF expression as well as the basal CTGF expression. This argued against a specific effect of spironolactone at the high concentration of 10−5 M. At lower concentrations, no inhibition was observed. In accordance with these results, two other specific antagonists of the MR, canrenoate or eplerenone, did not affect the aldosterone-induced expression of CTGF. These data strongly argue against an involvement of the MR in aldosterone-mediated upregulation of CTGF in mesangial cells. This notion was confirmed by the inhibitory effect of the specific antagonist of the glucocorticoid receptor RU486, which inhibited induction of CTGF in mesangial cells stimulated with either dexamethasone or aldosterone, without affecting the basal CTGF expression. The interaction between aldosterone and the GR was further confirmed by our finding that the GR was translocated to the nucleus in the presence of aldosterone. Specific interference of RU486 but not spironolactone with aldosterone has also been reported in liver cells in terms of aldosterone-mediated upregulation of gluconeogenic enzymes [31]. Our data thus indicated that aldosterone-induced CTGF expression was independent of the MR.

At a first glance, these data seem not to be in accordance with recently published reports, describing spironolactone-sensitive induction of CTGF by aldosterone in murine mesangial cells, proximal tubular cells [16] and vascular smooth muscle cells [17]. However, in these reports expression of CTGF was determined after 24 and 48 h, respectively. At these time points, CTGF expression can no longer be attributed solely to transcriptional regulation by a nuclear receptor but indirect regulation has to be taken into account. Induction of the SGK, which is a direct target of the MR [32], may be involved in long term expression of CTGF, because upregulation of cardiac CTGF by aldosterone was abolished in the heart tissue of SGK1 knock out mice [6]. Activation of MR in mesangial cells...
may thus indirectly contribute to CTGF expression, especially in the long-term induction of CTGF.

Our study supports a role for CTGF in aldosterone-mediated renal fibrosis, because pathophysiologically high concentrations of aldosterone contribute to CTGF induction in mesangial cells in vitro. However, our data argue against CTGF as a direct target gene of the MR, in line with aldosterone-induced CTGF expression under pathophysiological conditions but not in aldosterone-mediated homeostasis.

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References


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